Electrical Stimuli Release ATP to Increase GLUT4 Translocation and Glucose Uptake via PI3Kγ-Akt-AS160 in Skeletal Muscle Cells

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Skeletal muscle glucose uptake in response to exercise is preserved in insulin-resistant conditions, but the signals involved are debated. ATP is released from skeletal muscle by contractile activity and can autocrinely signal through purinergic receptors, and we hypothesized it may influence glucose uptake. Electrical stimulation, ATP, and insulin each increased fluorescent 2-NBDG uptake in primary myotubes, but only electrical stimulation and ATP-dependent 2-NBDG uptake were inhibited by adenosine-phosphate phosphatase and by purinergic receptor blockade (suramin). Electrical stimulation transiently elevated extracellular ATP and caused Akt phosphorylation that was additive to insulin and inhibited by suramin. Exogenous ATP transiently activated Akt and, inhibiting phosphatidylinositol 3-kinase (PI3K) or Akt as well as dominant-negative Akt mutant, reduced ATP-dependent 2-NBDG uptake and Akt phosphorylation. ATP-dependent 2-NBDG uptake was also inhibited by the G protein βγ subunit-interacting peptide (Gβct) and by the phosphatidylinositol 3-kinase-γ (PI3Kγ) inhibitor AS605240. ATP caused translocation of GLUT4greenfluorescent 2-NBDG to the cell surface, mechanistically mediated by increased exocytosis involving AS160/Rab8A reduced by dominant-negative Akt or PI3Kγ kinase-dead mutants, and potentiating by myristoylated PI3Kγ. ATP stimulated 2-NBDG uptake in normal and insulin-resistant adult muscle fibers, resembling the reported effect of exercise. Hence, the ATP-induced pathway may be tapped to bypass insulin resistance. Diabetes 62:1519–1526, 2013

Regular exercise confers numerous health benefits, including reduced risk of developing type 2 diabetes (1,2) and lowering blood glucose in people with diabetes, partly due to an increase in the rate of glucose transport into the skeletal muscles and elevating insulin sensitivity in the postexercise period (3–6). Despite the clinical importance of the metabolic effects of exercise, the underlying molecular mechanisms that mediate these responses remain unclear.

Exercise and insulin are the most important physiological stimuli to increase glucose transport into skeletal muscle fibers (5,7). Each stimulus results in redistribution of GLUT4 from the muscle cell interior to surface membranes, which ultimately leads to a larger glucose transport rate into the muscle cell (8–10). The proposed mechanisms through which a single bout of physical exercise increases glucose transport in skeletal muscle involve intracellular signal transduction that is distinct from that of insulin (5,11). The putative exercise-derived signals have long been elusive, but a need for intracellular Ca2+ below the threshold needed to evoke contraction was early recognized (12,13). AMP-activated protein kinase (AMPK) has been proposed as a mediator of insulin-independent glucose transport in skeletal muscle (14–16). Although AMPK is a master regulator of metabolic and transcriptional functions in tissues and cells throughout the body during exercise (17), its role in contraction-stimulated glucose transport has been questioned (18,19).

We previously reported that tetanic electrical stimulation of skeletal myotubes evokes a fast Ca2+ transient related to excitation–contraction coupling (20) and a slow Ca2+ transient, within seconds of stimulus initiation (21), that was not related to excitation-contraction coupling (22). The fast transient arises from Ca2+ release from ryanodine-sensitive stores, whereas the slow component is Ca2+ released through inositol-1,4,5-trisphosphate receptor (IP3R) (21,29) and depends on ATP concomitantly released from the stimulated cells (24). ATP signals in skeletal muscle through P2X and P2× purinergic receptors (24), G-protein-coupled receptors that typically signal through the βγ subunits to activate phosphatidylinositol 3-kinase-γ (PI3Kγ) (21). We therefore hypothesized that extracellular ATP may signal through PI3Kγ and further downstream to Akt. Because Akt phosphorylation is a key regulator of GLUT4 traffic, ATP may ultimately stimulate glucose uptake through this transporter. Here we describe that in primary and L6 myotubes as well as in adult muscle fibers, electrical stimulation and ATP indeed signal through PI3Kγ downstream to Akt and its substrate AS160 and mobilize GLUT4 to the surface via Rab8A-mediated exocytosis and decreased endocytosis to increase glucose uptake.

RESEARCH DESIGN AND METHODS

Reagents. ATP, ADP, UTP, and appargase grade VII from potato, suramin, cytosine arabinoside, penicillin-streptomycin, nifedipine, and amphotericin B were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium-F12, bovine serum, and FBS, were from Invitrogen (Grand Island, NY). Collagenase type II and IV were from Worthington Biochemical Corp. (Lakewood, NJ). Mini protease inhibitors were from Roche Applied Science
ATP IN ELECTRICALLY INDUCED GLUCOSE UPTAKE

(Manheim, Germany). Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were from Pierce Biotechnology (Rockford, IL). Enhanced chemiluminescence reagents were from Amersham Biosciences (Piscataway, NJ). Polyethyleneimine difluoride (PVPD) membranes were from Cell Signaling Technology (Danvers, MA). All other reagents and antibodies were from Sigma-Aldrich, Merck (Darmstadt, Germany), or Invitrogen. 2-NBD-Glucose (2-NBD-G) and anti-rabbit Alexa 546 were from Molecular Probes (Eugene, OR). L294002, Akt inhibitor VIII, and AS052540 were from Calbiochem (La Jolla, CA). Anti-myc polyclonal antibody was from Sigma-Aldrich. pAkt (Thr308, Ser473), anti-IRβ (Tyr1150/1151), 29160, and total AS160 were from Cell Signaling Technology (Danvers, MA). pSer341, pSer570, pSer588, and Thr 751 AS160 were provided courtesy of Dr. James Hastie (Division of Signal Transduction, University of Dundee). Rab8A and Rab10 (Santa Cruz Biotechnology, University of Dundee) were from Millipore (Bedford, MA). All other reagents were obtained from Sigma-Aldrich, Merck (Darmstadt, Germany), or Invitrogen. 2-NBD-Glucose hexose uptake was estimated by comparing intracellular concentrations of 5,000 at least 48 h before experiments. Western blotting was performed as previously described (25) and here termed the OPD assay (for o-phenylenediamine). Briefly, cells were stimulated and washed twice with ice-cold PBS, fixed with 4% (v/v) paraformaldehyde for 15 min, quenched with 100 mmol/L glycine for 10 min, and blocked with 5% (v/v) goat serum. Myotubes were incubated with polyclonal anti-IRβ antibody (1:150) for 2 h at 4°C, then washed with PBS and incubated with HRP-bound goat anti-rabbit secondary antibody (1:1000) for 1 h at 4°C. OPD reaction was carried out at room temperature. Glut4 endocytosis and re-endoxysosis. Glut4 myc internalization and re-endoxysosis were measured as previously described (20). Treatments with insulin and ATP were applied to cells before and after antibody labeling.

RESULTS

ATP detection by luciferase assay. Fifty microliters of extracellular samples with 290 μL CellTiter-Glo luminescence cell viability assay (Promega, Madison, WI). After a 10-min incubation in the dark, samples were quantified in a luminometer. In parallel, a standard curve from 1 nmol to 100 pmol ATP was performed using the same kit. Linearity was observed between 100 fmol and 10 pmol.

Recombinant adenosviruses. Adenoviruses (Ad) for myristoylated Akt (AdAkt-myr), and an empty construct (Ad-empty) were a gift from Dr. Joseph A Hill (University of Texas Southwestern Medical Center, Dallas, TX). Transduction efficiency was >90%, monitored with green fluorescent protein (GFP)-encoded recombinant adenovirus. Myotubes were infected with adenoviral vectors at a multiplicity of infection of 5,000 at least 48 h before experiments.

Cell-surface GLUT4 quantification. This assay was previously described (12) and here termed the OPD assay (for o-phénylenediamine). Briefly, cells were stimulated and washed twice with ice-cold PBS, fixed with 4% (v/v) paraformaldehyde for 15 min, quenched with 100 mmol/L glycine for 10 min, and blocked with 5% (v/v) goat serum. Myotubes were incubated with polyclonal anti-IRβ antibody (1:150) for 2 h at 4°C, then washed with PBS and incubated with HRP-bound goat anti-rabbit secondary antibody (1:1000) for 1 h at 4°C. OPD reaction was carried out at room temperature. Glut4 endocytosis and re-endoxysosis. Glut4 myc internalization and re-endoxysosis were measured as previously described (20). Treatments with insulin and ATP were applied to cells before and after antibody labeling.

Plasmids transfections and immunofluorescence microscopy. Myotubes were transfected with 3 μg Glut4-myc-GFP (29) PEK1-wild-type, PEK1-kine dead, PEK1-myr (donated by Dr. T.R. Jackson, University of Newcastle, U.K.) using 6 μLmL Lipofectamine 2000 (Invitrogen). Transfection efficiency was >90%. Myotubes were incubated with transfection mixture for 4 h in OPTI-MEM (Invitrogen) media, incubated in F12-Dulbecco’s modified Eagle’s medium (1:1) for 36 h, and stimulated and processed. Cell-surface Glut4-myc-GFP was determined as described (29).

Statistical analysis. Data were expressed as mean ± SD. Statistical differences between two groups of data were analyzed by Student t test. For multiple comparisons, one-way ANOVA was used, followed by Bonferroni post-test analysis, as appropriate. P < 0.05 was considered statistically significant.

Extracellular nucleotide measurement. The extracellular medium was replaced by 1.5 mL Krebs buffer 1 h before nucleotide assay. For whole-dish electrical stimulation, we built a device consisting of a row of six platinum wires 1 cm apart with alternate polarity across a circular plastic holder that fits the dish. Cells were stimulated with a tetanus protocol and extracellular aliquots were removed to 0 to 30 min poststimulus. Cells from each plate were lysed, and proteins were quantified using Coomassie Plus protein assay (Pierce, Rockford, IL). Data are expressed as picomoles of extracellular nucleotide per milligram of protein.

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RESULTS

Electrical stimulation-dependent 2-NBDG uptake requires purinergic receptors and extracellular nucleoside phosphate. Single myotubes analysis was performed to study the effects of electrical stimuli, ATP, and insulin on the uptake of the nonmetabolizable fluorescent glucose analog 2-NBDG (Fig. 1A). Moderate tetanic stimulation (45 Hz, 400 1-ms pulses lasting 9 s) induced an increase in 2-NBDG uptake (2.50 ± 0.55-fold), as did 100 μmol/L ATP (2.73 ± 0.52-fold) or 100 μmol/L insulin (2.87 ± 0.41; Fig. 1A and B). A significant increase was observed with 50 μmol/L external ATP (Supplementary Fig. 1A), and it peaked with 100 μmol/L. While primary myotubes were regularly grown in medium containing 25 mmol/L glucose, insulin, and ATP, similarly stimulated myotubes were cultured in 5.5 mmol/L (Supplementary Fig.1B). Exogenously added ADP or UTP also elevated 2-NBDG uptake (Supplementary Fig. 1C). Preincubation with 2 units/mL apyrase, which catalyzes the hydrolysis of both ATP and ADP, did not affect basal 2-NBDG uptake but eliminated the increase evoked by electrical stimulus (Fig. 1C). In addition, pretreatment with the purinergic receptor inhibitor suramin (100 μmol/L) inhibited electrical stimulation-dependent 2-NBDG uptake, without affecting basal uptake (Fig. 1D). Furthermore, apyrase (Fig. 1E) and suramin (Fig. 1F) both blocked ATP-dependent 2-NBDG uptake,
derived primary cultures of neither insulin nor ATP stimulated glucose uptake in muscle-activating Akt and the GAP-Rab AS160. Electrical stimulus increases extracellular ATP, levels by 1 min (Fig. 2A). Unlike insulin, exogenously added ATP did not affect insulin receptor phosphorylation (Supplementary Fig. 2A), indicating that ATP-induced glucose uptake occurs via an insulin receptor-independent pathway. However, downstream signals may be common to both insulin and ATP. Interestingly, tetanic stimulation of myotubes provoked an increase in Akt phosphorylation, and this response was abrogated by inclusion of suramin in the medium before and during stimulation (Fig. 2B). This finding suggests that purinergic receptors may be activated by electrical stimulation and lead to Akt activation. Maximal stimulation of Akt phosphorylation occurred 30 min after the electrical stimulation and decreased by 60 min (Supplementary Fig. 2D).

Exogenously added ATP also stimulated Akt phosphorylation at Thr308 and Ser473 to levels comparable to those induced by insulin (Fig. 2C). Insulin-activated Akt leads to phosphorylation of the GAP-Rab AS160, an event required for GLUT4 translocation. Like insulin, ATP strongly induced phosphorylation of AS160 at Thr751, Thr642, Ser588, Ser570, Ser341, and Ser318 (Fig. 2D). Moreover, exposing myotubes to electrical stimulation followed by insulin, produced an additive effect on Akt activation but not when the order was reversed (Fig. 2E). It is possible that the effect of a high dose of insulin may saturate the

without affecting insulin-dependent uptake (Supplementary Fig. 2F).

The stimulation of 2-NBDG uptake was corroborated in monolayers of myotubes using the conventional 3H-2DG uptake. Insulin or ATP each caused an increase in glucose uptake, measured after 20 min. Suramin strongly reduced ATP-dependent glucose uptake without affecting basal uptake (Supplementary Fig. 1A). Cytochalasin B, an inhibitor of class I GLUT activity (primarily GLUT1, 3, 4), completely abolished basal and insulin-dependent glucose uptake (Supplementary Fig. 1E), whereas Incluvirin, an effective GLUT4 inhibitor (30) also significantly decreased ATP-dependent glucose uptake, without affecting basal glucose uptake (Supplementary Figs. 1D and 2C). Of note, neither insulin nor ATP stimulated glucose uptake in muscle-derived primary cultures of fibroblasts (data not shown).

**Electrical stimulus increases extracellular ATP, activating Akt and the GAP-Rab AS160.** We measured ATP in the medium after tetanic stimulation. A transient ATP increase was significant by 30 s and reached maximal levels by 1 min (Fig. 2A). Unlike insulin, exogenously added ATP did not affect insulin receptor phosphorylation
same Akt pool that is targeted by electrical stimulation, whereas tetanic stimulation does not. Further experiments are needed to ascertain whether a fast turnover rate of phosho-Akt after electrical stimuli or recruitment of an additional pool could explain the additive effect of insulin in these conditions.

**ATP-dependent glucose uptake involves the PI3K-Akt axis.** The general PI3K inhibitor, LY-294002 (50 μmol/L) abrogated the effect of insulin but only partly reduced ATP-induced Akt phosphorylation (Supplementary Fig. 2D). Similarly, LY-294002 eliminated the stimulation by insulin of 2-NBDG uptake but incompletely reduced ATP-dependent stimulation by 70% (Fig. 3A). Unlike a more complete inhibition of insulin action, the specific Akt inhibitor Akt1/2 (10 μmol/L) only partially inhibited ATP-dependent 2-NBDG uptake (Fig. 3B). These results suggest that ATP and insulin do not trigger identical signaling mechanisms.

The participation of Akt was further investigated by transducing primary myotubes with adenovirus encoding AdAkt-dn [multiplicity of infection (MOI) 5,000] or, as positive control, constitutively active AdAkt-myr (MOI 5,000). Empty vector was used as transduction control (Ad-empty, MOI 5,000). The ATP-dependent 2-NBDG uptake recorded in myotubes transduced with Ad-empty was similar to that in nontransduced cells. In contrast, myotubes infected with AdAkt-dn exhibited a decrease in the ATP response. Overexpression of Akt-myr elevated basal 2-NBDG uptake sevenfold over the nontransfected control, and ATP did not have a further stimulatory effect (Fig. 3C).

**ATP promotes GLUT4 translocation to the cell surface.** To visualize GLUT4 exposure at the plasma membrane, we transiently transfected cDNA encoding a GLUT4myc-eGFP chimera. Confocal slices (0.7 μm) and whole cell z stacks were imaged. In the basal state, myotubes showed GLUT4myc vesicles in the cytoplasm and perinuclear regions with a low surface myc epitope signal (Fig. 4A). By comparison, ATP-stimulated myotubes showed an increase in exocortically exposed myc epitope (Fig. 4A), akin to that evoked by insulin (data not shown). Cotransfection with Akt-dn plasmid lowered this gain without affecting the basal levels of surface GLUT4myc-eGFP (Fig. 4A). ATP increased the myc-eGFP ratio by 2.50 ± 0.3 relative to nonstimulated cells, whereas the basal myc-eGFP ratio was 0.82 ± 0.06 in Akt-dn cotransfected myotubes, and ATP raised this value to only 1.48 ± 0.15-fold above the basal control (Fig. 4B). However, cotransfection of Akt-myr plasmid evoked a maximal GLUT4myc surface exposure in nonstimulated myotubes. ATP-stimulated myotubes did not show any additional increase of surface myc epitope, indicating that the response had peaked (data not shown). These results suggest that ATP causes GLUT4myc translocation to cell surface by a mechanism that involves Akt activation.

**ATP-dependent glucose uptake and GLUT4 translocation in myotubes require PI3Kγ activation.** Overexpression of Bαrck-ct (inhibitor of βγ subunits) partially abolished ATP-dependent 2-NBDG uptake (Fig. 5A), and AS 605240, a specific inhibitor of PI3Kγ, efficiently inhibited the ATP effect (Fig. 5A). We cotransfected GLUT4myc-eGFP along with different PI3Kγ mutants to evaluate the participation of PI3Kγ in GLUT4 translocation to the cell surface. Myotubes cotransfected with wild-type PI3Kγ and stimulated with ATP show a significant increase in surface GLUT4myc compared with control, ATP-stimulated cells (Fig. 5B). Conversely, cotransfection of GLUT4myc with ATP was detected by immunofluorescence in nonpermeabilized cells as described in RESEARCH DESIGN AND METHODS. Z stack images were collapsed in a single slice (z projection) and results expressed as the ratio of the fluorescence of surface-labeled GLUT4myc (red, Alexa 546) to the fluorescence of total GLUT4 expressed (green GFP). A: Exogenous ATP induced an increase in the Alexa 546-to-eGFP ratio. This ratio was reduced significantly in myotubes cotransfected with GLUT4myc-eGFP and Akt-dn. B: Quantification of panel A. Values are the mean ± SD. **P < 0.001 vs. basal group; ††P < 0.001 relative to ATP-stimulated and Akt-dn.
the kinase dead form of PI3K strongly decreased the effect of ATP on surface GLUT4myc (Fig. 5C). Finally, in myotubes cotransfected with PI3K-myr, a myristoylated (constitutively active) form of PI3Kα, the surface GLUT4myc signal was increased in basal relative to the control condition, and as in Fig. 5B, the stimulating effect of ATP is still seen, possibly indicating that a larger PI3K pool is present in these conditions (Fig. 5D).

**ATP signaling downstream of Akt toward AS160 and Rab8A.** In response to insulin, Akt-AS160 phosphorylation allows manifestation of the activity of its target Rab guanosine triphosphatases leading to GLUT4 translocation to the membrane. The pertinent RabS in L6 muscle cells are Rab8A and Rab13 (31), and Rab10 prevails in 3T3-L1 adipocytes (32,33). Because AS160 is phosphorylated in response to ATP in muscle cells, we examined the possible participation of its target RabS in ATP-mediated glucose uptake. In L6 muscle cells stably expressing GLUT4myc (L6-GLUT4myc), ATP again caused an increase in the exofacial exposure of the myc epitope that was significant by 2 min and achieved a comparable level to that evoked by insulin (Supplementary Fig. 3). Knockdown of Rab8A by 80% caused significant inhibition of insulin- or ATP-dependent GLUT4myc translocation to cell surface (Fig. 6A and B). In contrast, similar knockdown of Rab10 did not affect the insulin- or ATP-dependent GLUT4myc translocation (Fig. 6C). Together, these data suggest participation of the Akt-AS160-Rab8A axis in ATP-dependent GLUT4 translocation and glucose uptake.

**ATP-dependent GLUT4 translocation to cell surface involves a rise in GLUT4 exocytosis and a drop in GLUT4 endocytosis.** To ascertain if the increase in surface GLUT4myc arises from exocytic vesicles similar to those promoted by insulin, primary myotubes were transiently cotransfected with GLUT4myc-eGFP and tectanus toxin light chain (TeTx). This toxin proteolytically cleaves vesicle-associated membrane protein 2 (VAMP2), a key v-SNARE mediating docking and fusion of insulin-sensitive GLUT4-storage vesicles (GSV) with the plasma membrane (25,34). Under basal conditions (absence of ATP), surface GLUT4myc was practically undetectable (Fig. 7A). The whole-cell quantification of GLUT4myc is shown for each condition (Fig. 7B). ATP caused a clear increase in surface myc epitope signal, and this response diminished in myotubes expressing TeTx (Fig. 7A).

The rates of exocytosis and endocytosis of GLUT4myc were investigated in L6-GLUT4myc muscle cells, a system amenable to these determinations because they require large numbers of homogeneously labeled cells (25). ATP reduced GLUT4myc endocytosis compared with insulin-stimulated or untreated cells (Fig. 7C). The rate of exocytosis was then determined after allowing surface GLUT4myc loaded with anti-myc antibody to internalize and equilibrate with intracellular GLUT4 pools before insulin stimulation. ATP caused a significant increase in GLUT4myc externalization, noticeable by 2 min, resembling the effect of insulin (Fig. 7D). Exocytosis likely involves GSV that rely on VAMP2 for fusion, as is the case of insulin-regulated GSV. Exogenous ATP increase 2-NDBG uptake in adult muscle fibers from normal and insulin-resistant mice. ATP (100 μmol/L) induced a significant increase in 2-NDBG uptake in adult fibers isolated from flexor digitorum
breviss muscles; a similar increase was obtained with 100 nmol/L insulin (Fig. 8A). Although neither 10 μmol/L ATP nor 10 nmol/L insulin induced a significant increase, a synergistic effect was evident when applied together (Fig. 8A). Interestingly, in muscle fibers obtained from mice fed a high-fat diet, the effect of 100 nmol/L insulin on glucose uptake was not present, but 100 μmol/L ATP produced a significant increase, similar to that of muscle fibers from mice fed the control diet (Fig. 8B).

**DISCUSSION**

We describe here a novel mechanism for GLUT4 translocation and glucose uptake that depends on extracellular ATP released by cultured muscle cells after electrical stimulation (Fig. 8C). This stimulus acts via action potentials (23), mimicking the physiological activation of skeletal muscle during exercise. Electrical stimulation is able to elicit a cascade of events triggered by ATP release and binding to P2Y purinergic receptors, followed by activation of the serine/threonine kinase Akt that depends on PI3K. Each of the above-mentioned elements is required for ATP-dependent GLUT4 translocation, revealing an unrealized connection between electrical stimulation and Akt activation that can now be studied in the context of exercise. ATP-dependent GLUT4 translocation to the cell surface is related to an increase in the transporter’s exocytosis and a decrease in its endocytosis.

The muscle interstitial ATP concentration increases with muscle contraction (35), and muscle contraction per se is a necessary and sufficient stimulus to significantly raise extracellular ATP (35). Electrical stimulation of the ventral roots increased the dialysate ATP concentration in contracting muscle by 150% (35), reaching micromolar concentrations. The somewhat higher concentration of exogenous ATP required to stimulate glucose uptake is probably needed because ectonucleotidases present in the T-tubule membrane can significantly reduce the effective levels.

Mild electrical stimulation was recently reported to activate PI3K/Akt signaling and increase glucose uptake in L6 and C2C12 muscle cells (36,37). We here show that exogenously added ATP leads to phosphorylation of the two activator sites on Akt. Contractile activity does not result in tyrosine phosphorylation of IRS1 or associated class I PI3K activation (38) but can lead to Akt phosphorylation (39). Contraction-induced Akt activation can be inhibited by wortmannin or LY294002, consistent with a PI3K-dependent process (7). However, contraction does not activate class IA PI3K (7,38) or class II PI3K (40). It is possible that contraction might activate Akt through class

**FIG. 7.** ATP-dependent GLUT4myc translocation requires VAMP2 and reduces GLUT4myc endocytosis. A and B: Primary myotubes were transiently cotransfected with GLUT4myc-eGFP and TeTx cDNA for 24 h and then stimulated with 100 μmol/L ATP for 10 min. C: GLUT4myc endocytosis in L6-GLUT4myc cells. Surface GLUT4myc was labeled with anti-mycc antibody, followed by extensive washing with PBS. The surface-labeled GLUT4myc was then allowed to internalize by re-warming for different times in presence or absence of the respective stimulus. At the indicated times, the amount of GLUT4myc remaining at the cell surface was determined and expressed as a percentage of the cell surface level at 0 min of endocytosis. **DISCUSSION**

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IB PKγ, an enzyme that is activated by heterotrimeric G-protein–coupled receptors rather than tyrosine kinase–coupled receptors (discussed in 41). Our study shows that ATP engages PI3Kγ to mediate its downstream effect on glucose uptake. PKγ is a well-known target of G-coupled receptors, consistent with ATP signaling via purinergic G-coupled receptors. Indeed, exogenous nucleotides lead to Ca2+ mobilization, ERK activation, and gene expression in skeletal muscles through activation of PK2/P2Y receptors (42, 43).

A further finding of the current study is that exogenous ATP causes phosphorylation of the Akt substrate AS160. This Rab-GAP is believed to be inactivated upon phosphorylation by upstream kinases, allowing activation of Rab proteins required for GLUT4 translocation to the plasma membrane (31). This pathway has been mapped for insulin-stimulated GLUT4 translocation in insulin-sensitive 3T3-L1 adipocytes (44) and L6 myoblasts (25). In addition to its COOH-terminal Rab-GAP domain, AS160 has six phosphorylation motifs (RXRXXS*/T*) targeted by, but not exclusively, Akt: Ser318, Ser341, Ser570, Ser588, Thr642, and Ser751 (45, 46). Interestingly, here we show that ATP induces phosphorylation of the very same residues. AS160 phosphorylation by AMP-kinase occurs predominantly on residues Ser630, Ser711, Ser761, Ser764, and Ser1135 (47). Although contraction stimulated AS160 phosphorylation at Ser318, Ser341, and Ser751, with a tendency to also increase Ser588, it did not have any evident effect on Thr642 or Ser666 (48). In contrast, ATP led to phosphorylation of Thr642 in the current study, suggesting that more than one pathway is involved in AS160 phosphorylation.

AS160 and the related protein TBC1D1 have been proposed as potential sites for the convergence of insulin and exercise signaling leading to stimulation of glucose transport in skeletal muscle (11, 49, 50). It is tantalizing to hypothesize that ATP may be an element conveying the insulin-sensitizing effect of exercise by increasing AS160 phosphorylation. We could not study phosphorylation of TBC1D1 because its expression in cultured myotubes is too low to be detected by currently available antibodies.

A further novelty of this study is the revelation that ATP largely promotes GLUT4 exocytosis and partly reduced its endocytosis. The exocytic route likely involves GSV that fuse via TeTx-sensitive SNAREs, such as VAMP2, and GSV traffic is promoted by Rab8A but not Rab10.

Exercise is a potent stimulus that enhances insulin action in skeletal muscle from diabetic patients, and considering that effects of ATP seen in myotubes were also seen in adult muscle fibers and are still present in an insulin resistance model, this study invites the possibility that extracellular ATP or the signaling pathway it enacts may be tapped upon to design strategies to treat diabetic insulin resistance.

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C.O.-F. and A.E.C.-F. researched data, wrote the manuscript, and approved the final version. F.A. and Q.L. researched data and approved the final version of the manuscript. A.E. contributed to the study design, to analysis and interpretation of data, to drafting and revising the manuscript, and approved the final version. W.N. contributed to the analysis and interpretation of data and approved the final version of the manuscript. S.L. contributed to the study design, to analysis and interpretation of data, and approved the final version of the manuscript. A.K. contributed to the study design, to analysis and interpretation of data, to revising the manuscript, and approved the final version of the manuscript. E.J. contributed to the study design, to analysis and interpretation of data, to writing and revising the manuscript, and approved the final version of the manuscript. E.J. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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