Calcium signaling recruits substrate transporters GLUT4 and CD36 to the sarcolemma without increasing cardiac substrate uptake

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Calcium signaling recruits substrate transporters GLUT4 and CD36 to the sarcolemma without increasing cardiac substrate uptake. Am J Physiol Endocrinol Metab 307:E225–E236, 2014. First published June 3, 2014; doi:10.1152/ajpendo.00655.2013.—Activation of AMP-activated protein kinase (AMPK) in cardiomyocytes activates translocation of glucose transporter GLUT4 and long-chain fatty acid (LCFA) transporter CD36 from endosomal stores to the sarcolemma to enhance glucose and LCFA uptake, respectively. Ca2+ /calmodulin-activated kinase β (CaMKKβ) has been positioned directly upstream of AMPK. However, it is unknown whether acute increases in [Ca2+]i stimulate translocation of GLUT4 and CD36 and uptake of glucose and LCFA or whether Ca2+ signaling converges with AMPK signaling to exert these actions. Therefore, we studied the interplay between Ca2+ and AMPK signaling in regulation of cardiomyocyte substrate uptake. Exposure of primary cardiomyocytes to inhibitors or activators of Ca2+ signaling affected neither AMPK-Thr172 phosphorylation nor basal and AMPK-mediated glucose and LCFA uptake. Despite their lack of an effect on substrate uptake, Ca2+ signaling activators induced GLUT4 and CD36 translocation. In contrast, AMPK activators stimulated GLUT4/CD36 translocation as well as glucose/LCFA uptake. When cardiomyocytes were cotreated with Ca2+ signaling and AMPK activators, Ca2+ signaling activators further enhanced AMPK-induced glucose/LCFA uptake. In conclusion, Ca2+ signaling shows no involvement in AMPK-induced GLUT4/CD36 translocation and substrate uptake but elicits transporter translocation via a separate pathway requiring CaMKK activity. Ca2+-induced transporter translocation by itself appears to be ineffective to increase substrate uptake but requires additional AMPK activation to effectuate transporter translocation into increased substrate uptake. Ca2+-induced transporter translocation might be crucial under excessive cardiac stress conditions that require supraphysiological energy demands. Alternatively, Ca2+ signaling might prepare the heart for substrate uptake during physiological contraction by inducing transporter translocation. Ca2+/calmodulin-activated kinases; AMP-activated protein kinase; glucose transporter 4; CD36; cardiomyocytes

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[Ca^{2+}], in the heart is finely regulated and the activity of CaMKs regulated by protein phosphatases in concert with [Ca^{2+}]i (3, 6, 8). Despite the fact that both CaMKK and CaMKII are activated in response to a cytoplasmic Ca^{2+} rise via a Ca^{2+}/calmodulin-dependent manner, it has been shown in skeletal muscle that both kinases also have autonomous activity (16, 38). Additionally, it was reported that exercise leads to rapid activation and increased phosphorylation of CaMKII and its downstream targets (37). However, the degree of autonomous activity of both CaMKKs and CaMKs, the speed of their activation, and the duration of their active states have not yet been reported for heart tissue.

Studies describing the role of Ca^{2+} signaling in skeletal muscle substrate uptake often have used caffeine to increase [Ca^{2+}]i (1, 48). Specifically, it has been shown that caffeine treatment induces CaMKKβ and CaMKII phosphorylation concomitantly with increased glucose uptake. This caffeine-induced glucose uptake was sensitive to pharmacological CaMKKβ and CaMKII inhibition (48). Moreover, contraction-induced glucose uptake was at least partly inhibited by CaMKKβ and CaMKII inhibitors, which was indicative of a role for both Ca^{2+}-activated kinases in contraction-induced glucose uptake (48). Contraction-induced LCFA uptake also appeared to be inhibited by CaMKKβ inhibition in skeletal muscle (2), whereas CaMKII inhibition has not yet been studied in this respect.

In contrast to skeletal muscle, information is lacking about the roles of CaMKKβ and CaMKII in contraction-induced substrate uptake in the heart, especially in relation to AMPK. A possible bridge between Ca^{2+} signaling and AMPK signaling might be provided by CaMKKβ, because it is one of the kinases known to have AMPK kinase activity in vitro and to activate AMPK in mammalian cells deficient in LKB1 (25, 47). Indeed, several studies in skeletal muscle indicate that CaMKKβ and CaMKII stimulate muscle substrate uptake through AMPK activation (2, 28). However, other data suggest that this Ca^{2+}-induced signaling axis stimulates substrate uptake independently of AMPK (46, 48). Therefore, the aim of this study was to investigate the interrelation between AMPK, CaMKs, and [Ca^{2+}]i in the regulation of transporter-mediated substrate uptake into cardiomyocytes. To assess separately the effects of [Ca^{2+}]i, and of CaMKKβ/CaMKs on AMPK-mediated substrate uptake, we used pharmacological agents to activate these signaling pathways individually and independently from contraction.

MATERIALS AND METHODS

Animals

Rats were maintained at the Experimental Animal Facility of Maastricht University. All study protocols involving the rat experiments were approved by the Animal Care and Use Committee of Maastricht University and were performed according to the official rules formulated in the Dutch law on care and use of experimental animals, which are highly similar to those of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Materials

2-Deoxy-d-[1-3H]glucose and [1-14C]palmitic acid were obtained from GE Healthcare (Piscataway, NJ). Bovine serum albumin (BSA; fraction V, essentially fatty acid free), laminin, phloretin, DMSO, thapsigargin, A23187, STO-609, KN93, oligomycin, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), and adenosine 9-β-D-arabinofuranoside (ara-A) were obtained from Sigma–Aldrich (St. Louis, MO). Sulfo-NHS-LC-biotin and immobilized streptavidin were from Perbio Science (Etten-Leur, The Netherlands). Compound C was from Calbiochem (Radnor, PA). Flu-4 acetoxy-methylester and pluronidic acid (F1420c1) were from Invitrogen (Bleiswijk, The Netherlands).

Antibodies

Antibodies were purchased as indicated: phospho-ACC (Ser79; no. 07-330) from Upstate (Billerica, MA); phospho-AMPKα (Thr172; no. 2531), phospho-AS160 (Thr642; no. 4288), and phospho-CaMKII (Thr286; no. 3361) from Cell Signaling Technology (Beverly, MA); phospho-CaMKI (sc-28438-R) from Santa Cruz Biotechnology (Santa Cruz, CA); caveolin-3 (no. 610420) from BD Transduction Laboratories (Franklin Lakes, NJ); anti-CD36 antibody (CRF D-2717) used in 2-photon microscopic images from BD Pharmingen (Franklin Lakes, NJ); and anti-GLUT4 (Ab 1346) used in Western Blotting from Chemicon International (Billerica, MA). FITC-labeled rabbit anti-mouse IgG secondary antibody was from Rockland Immunokemical (Gilbertsville, PA). The anti-CD36 antibody (no. MO25) used in Western Blotting was a gift from Dr. N. Tandon (Bethesda, MD).

Isolation of Primary Rat Cardiomyocytes

Adult rat cardiomyocytes (Lewis rats; 200–250 g, 2–3 mo of age) were isolated by using a Langendorff perfusion system according to the procedure developed by Fischer et al. (12), as has been described previously (33). This method yields mainly ventricular myocytes. A modified Krebs-Henseleit bicarbonate (MKR) medium was stored at a 10-fold concentration and contained 1.17 M NaCl, 26 mM KCl, 12 mM KH2PO4, 12 mM MgSO4, 100 mM NaHCO3, and 100 mM HEPES. Upon 10-fold dilution of the concentrated stock for daily use, the MKR medium was adjusted to pH 7.55 and equilibrated with a 95% O2-5% CO2 gas phase at 37°C.

Experiments With Primary Cardiomyocytes

To recover from the isolation procedure, cardiomyocytes were incubated for an additional 90 min in medium A (1 × MKR medium supplemented with 2% BSA, 2 mM D-glucose, and 1 mM CaCl2) while rotating at room temperature (24°C). For substrate uptake measurements ~200,000 cells/condition were used, and for signaling experiments ~100,000 cells/condition were used. During these experiments, cell suspensions were incubated with/without stimulators and/or inhibitors of Ca^{2+} and AMPK signaling in capped 20-ml glass vials. The vials were placed in a 37°C water bath under continuous shaking at 160 rpm.

Measurement of 2-Deoxy-[1-3H]glucose and [1-14C]palmitate Uptake Rates Into Cardiomyocytes

To increase [Ca^{2+}], and also activate CaMKs, cardiomyocytes were exposed to 5 μM A23187 or 5 μM thapsigargin for 20 min. For AMPK activation, cardiomyocytes were treated with either oligomycin (5 μM) or AICAR (1.5 mM) for 20 min or subjected to 4-Hz electric field stimulation during the last 7 min of a 20-min total of incubation time. For inhibition of CaMKs by STO-609 (5 μM) or KN93 (5 μM), cardiomyocytes were preincubated for 20 min, whereafter either CaMKs or AMPK activating stimuli were added for an additional 20 min. Subsequently, substrate uptake was measured by the addition of 0.5 μl of a mixture of a 2-deoxy-[1-3H]glucose and [1-14C]palmitate/BSA complex, as described previously (33). Five minutes after the addition of the radiolabeled substrates, the uptake reaction was stopped by transferring the cell contents to 15-ml Falcon tubes containing ice-cold MKR buffer with 0.1% BSA and 0.2 mM phloretin (stop solution). Cells then were washed twice for 2 min.
at 45 g in ice-cold stop solution, as described previously (33). The radioactivity of the cell pellets was measured by scintillation counting.

**Measurements of $[Ca^{2+}]_i$**

Freshly isolated cardiomyocytes were plated in laminin-coated 12-well plates in medium A. Cardiomyocytes were allowed to attach to the bottom of each well for 90 min before loading with 5 μM fluo-4 acetoxyethyl ester (AM) and 2 mg/ml pluronic F-127 for 30 min. After a 15-min period to allow probe deesterification, the fluo-4-loaded cells were washed three times with medium A. The washed cells in the 12-well plate were subjected to $[Ca^{2+}]_i$ measurements by pseudo-ratio fluorescence microscopic imaging using a filter wheel-controlled Nikon Diaphot 200 microscope equipped with a Hamamatsu EM-CCD digital camera and VitiTech (Sunderland, UK) image control software (26). Fluorescence image recordings at 0.5 Hz from microscopic fields (capturing for 20 ms) containing at least 15 cardiomyocytes were performed during the addition of thapsigargin, A23187, or oligomycin (each at 5 μM) at the indicated time periods. Where indicated, cardiomyocytes were subjected to electric field stimulation at a frequency of 4 Hz; image recording was at 0.24-s time intervals. Fluo-4 fluorescence was analyzed per region of interest per image, representing a single cell, and corrected for background (adjacent regions of interest not representing a cell). Changes in fluorescence per region of interest were converted to pseudo-ratio values $F' = F/F_0$, where $F$ is the measured fluorescence value, $F_0$ is the average fluorescence level for 20 s under resting conditions, and $F'$ is the pseudo-ratio fluorescence level at any time (4).

**Surface Detection of GLUT4 and CD36**

Three distinct methods were used to assess cellular surface presence of GLUT4 and CD36.

**Biotinylation method.** The biotinylation technique was used to separate plasma membrane proteins from subcellular proteins. After isolation, cardiomyocytes were plated on laminin-coated (10 μg/ml) 35-mm culture plates. After a 90-min attachment, cells were incubated with stimulants and subsequently biotinylated with the cell-impermeable reagent sulfo-NHS-LC-biotin in 1× MCK medium at a final concentration of 1 mg/ml for 45 min at 4°C, as described previously (43). Thereafter, cells were treated with ice-cold glycine (100 mM) in 1× MCK medium. After a brief wash with ice-cold 1× MCK medium, cells were scraped in 300 μl of lysis buffer (consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% (vol/vol) Igepal CA-630 (or NP-40), 5% (wt/vol) sodium deoxycholate, 1% (wt/vol) SDS, 4% (vol/vol) complete protease inhibitor cocktail, 5% PhosSTOP phosphatase inhibitor cocktail). The lysates were rotated for 1 h at 4°C and centrifuged for 10 min at 13,000 g at 4°C. Thirty microliters of supernatant was used for detecting total protein samples with Western blotting, and 150 μl of supernatant was incubated overnight with streptavidin beads. Samples then were centrifuged for 2 min at 13,000 g at 4°C. Thereafter, beads were washed twice with lysis buffer. The biotinylated proteins were eluted by incubation of the streptavidin beads for 5 min at 95°C in sample buffer (40% glycerol, 0.25 M Tris, 1 M DTT, bromphenol blue). Samples were subjected to SDS polyacrylamide gel electrophoresis, followed by Western blotting for the detection of GLUT4 and C36.

**Fractionation method.** After recovery, cardiomyocytes were pretreated for 20 min with kinase inhibitors STO-609 and KN93 and subsequently stimulated with the indicated agonists for 15 min. Thereafter, cell suspensions were subjected to the fractionation procedure, as described previously (32). GLUT4 and CD36 proteins were detected by Western blotting.

**Two-photon microscopy.** Following a 10-min pretreatment with either A23187, thapsigargin, or oligomycin, cells were labeled for 10 min with 2 μg/ml (diluted in medium A) anti-CD36 antibody. Subsequently, FITC-labeled rabbit anti-mouse IgA secondary antibody (C_end 1:500) was added to the cells. Finally, cardiomyocytes were washed twice with medium A. Viable cardiomyocytes were imaged using a Leica SP5 Multiphoton imaging platform (Leica Microsystems). The excitation wavelength of the 140 fs-pulsed laser was 800 nm, whereas emission filters were optimized for FITC detection (500–560 nm). Laser power was kept as low as possible to avoid bleaching and photo damage. Because antibodies do not have access to the cellular interior in these intact cardiomyocytes, only cell surface CD36 will be stained. For quantification of the cell surface staining, images were processed with ImageJ software (JAVA-based imaging software from the National Institutes of Health). Cardiomyocytes were kept at 37°C during incubations and imaging.

**Statistics**

Differences among the data obtained from five to eight experiments are presented as means ± SE. Statistical differences between groups of observations were evaluated by unpaired Student’s t-test, one-way ANOVA, and/or two-way ANOVA, depending on the groups compared, by using statistical analysis software Prism 5 (GraphPad Software). A P value ≤0.05 was considered statistically significant.

**RESULTS**

**Testing the Potential of Thapsigargin and A23187 as $[Ca^{2+}]_i$ Elevating Agents and STO-609 and KN93 as Ca$^{2+}$ Signaling Inhibitors in Cardiomyocytes**

Fluorescent Ca$^{2+}$ indicators are suitable tools for studying Ca$^{2+}$ transients in primary adult cardiomyocytes (23). In the present study, cardiomyocytes isolated from adult rats were adhered in 12-well plates and loaded with the Ca$^{2+}$ probe fluo-4 to measure single-cell [Ca$^{2+}]_i$ transients by microscopic fluorescence imaging. We used two agents with different action mechanisms, i.e., the sarco/endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor thapsigargin and the Ca$^{2+}$ ionophore A23187, to study the effects of increased [Ca$^{2+}]_i$. Each of these agents was used at 5 μM, in agreement with previous studies in myocytes (24, 44). Under resting conditions, in the absence of electrical field stimulation, the majority of cells displayed incidental Ca$^{2+}$ transients (Fig. 1), which paralleled spontaneous contractions of the cells. In fluo-4-loaded cells treated with the ATP synthase inhibitor oligomycin, the frequency of the Ca$^{2+}$ transients increased threefold. In these oligomycin-treated cells, as well as in cells exposed to 4-Hz electrostimulation, levels of [Ca$^{2+}]_i$ fully restored to basal levels in between each transient (Fig. 1). In contrast, stimulation of the cells with either thapsigargin or A23187 provoked marked and prolonged increases in [Ca$^{2+}]_i$, not restoring to baseline values within 5 min. At these concentrations, A23187 and thapsigargin did not affect short-term cell survival, as the rod-shaped appearance of the cardiomyocytes was not altered by these treatments (Fig. 1).

Compatible with their ability to increase [Ca$^{2+}]_i$, thapsigargin and A23187 enhanced phosphorylation of CaMKI (1.6- and 2.2-fold, respectively) and of CaMKII (1.4- and 1.6-fold, respectively) (Fig. 2). A23187-induced CaMKI phosphorylation was significantly inhibited by KN93 but not by STO-609 (Fig. 2). In contrast, A23187-induced CaMKII phosphorylation was significantly inhibited by KN93 but not by STO-609 (Fig. 2). Both inhibitors were used at 5 μM, in agreement with previous cell studies (25, 45). We did not observe an increased phosphorylation of CaMKI or of CaMKII upon 7-min, 4-Hz stimulation (Fig. 2B), which is in contrast to findings in skeletal...
Additionally, treatment with oligomycin or AICAR treatment did not increase phosphorylation of either kinase (Fig. 2B).

We conclude that thapsigargin and A23187 increase $[Ca^{2+}]_i$, thereby activating both CaMKKβ- and CaMKII-dependent signaling events in cardiomyocytes. Furthermore, Ca$^{2+}$ signaling remains unchanged in response to oligomycin or AICAR, indicating their specificity for AMPK activation in our cellular model. Also, the inhibitors STO-609 and KN93 block their intended target enzymes CaMKKβ and CaMKII, respectively.

**Effects of Ca$^{2+}$ Signaling Inhibitors on AMPK Activation and on AMPK-Mediated Substrate Uptake in Cardiomyocytes**

We investigated the roles of CaMKKβ and CaMKs in AMPK activation and in AMPK-mediated substrate uptake into cardiomyocytes. To activate AMPK in cardiomyocytes, we used both a pharmacological approach (oligomycin or AICAR) and a physiological approach (4-Hz electrostimulation). Each of these treatments induced AMPK-Thr$^{172}$ phosphorylation (7.4-, 6.2-, and 2.9 -fold, respectively) and, concomitantly, phosphorylation of AMPK’s major substrate ACC at Ser$^{97}$ (10-, 8-, and 3-fold, respectively) (Fig. 3A), in agreement with our previous observations (32, 34). Correspondingly, all three AMPK activators enhanced palmitate uptake significantly (1.7-, 1.3-, and 1.4-fold, respectively). Additionally, oligomycin and 4-Hz electrostimulation enhanced deoxyglucose uptake (2.2- and 1.4-fold, respectively), whereas AICAR showed no effect (Fig. 3A). The inability of AICAR to induce glucose uptake is in agreement with our previous observations (10, 19, 33) and relates to the fact that, next to AMPK activation, glucose uptake requires...
additional activation of protein kinase D1. As a result, AICAR, which in contrast to oligomycin or 4-Hz stimulation does not activate protein kinase D1, can only be used to study AMPK-mediated LCFA uptake (10). Pretreatment of cardiomyocytes with STO-609 or KN93 did not alter AMPK-Thr172 and ACC-Ser97 phosphorylation induced by all three AMPK-activating stimuli (Fig. 3A). Furthermore, pretreatment with either of these inhibitors did not alter the effect of each of the three AMPK-activating stimuli on deoxyglucose or palmitate uptake (Fig. 3B).

In conclusion, Ca\(^{2+}\)/\(H_{11001}\)signaling does not appear to be involved in either in AMPK activation or in AMPK-mediated substrate uptake into cardiomyocytes.

Effects of Ca\(^{2+}\)-Elevating Stimuli on GLUT4 and CD36 Translocation in Cardiomyocytes

To investigate the effect of thapsigargin and A23187 on GLUT4 and CD36 translocation, we used three independent methods to monitor transporter translocation: cell surface biotinylation, subcellular fractionation, and immunolabeling. Immunolabeling of cell surface protein, in contrast to the other methods, can be employed only for CD36 and not for GLUT4 detection, because suitable GLUT4 antibodies that are capable of recognizing the cell surface epitopes are lacking at present. First, we examined GLUT4 and CD36 translocation via cell surface biotinylation with sulfo-NHS-LC-biotin, which binds to free amino groups of extracellularly exposed lysine residues. We have already applied this method successfully to monitor insulin-induced CD36 translocation in Chinese hamster ovary cells (43). This method is also suitable for detecting cell surface GLUT4, notwithstanding that GLUT4 expresses only a single extracellular lysine (at position 50). Accordingly, we (22) and others (11, 42) have shown that insulin stimulates the binding of sulfo-NHS-LC-biotin to GLUT4. In the present study, oligomycin, a well-established inducer of GLUT4 and CD36 translocation (32), was used as positive control to validate the suitability of the cell surface biotinylation protocol and also of the other methods to detect surface GLUT4 and CD36. As shown in Fig. 4A, the biotinylation experiments showed that oligomycin, thapsigargin, and A23187 enhanced sarcolemmal contents of GLUT4 (1.3-fold each) and CD36 (1.8-, 2.1-, and 1.9-fold, respectively) (Fig. 4A). This increase in cell surface content of both transporters occurred in the absence of changes in overall protein expression of GLUT4 and CD36 (Fig. 4A). Using subcellular fractionation, we also detected an increased sarcolemmal content of GLUT4 (1.5-, 1.3-, and 1.4-fold, respectively) and of CD36 (1.5-, 1.7-, and 1.4-fold, respectively) upon treatment with each of these stimuli (Fig. 4B). This method further demonstrated a

Fig. 2. Effects of the selected Ca\(^{2+}\) signaling activators and inhibitors as well as AMP-activated protein kinase (AMPK) activators on phosphorylation of Ca\(^{2+}\)-dependent protein kinases. A: cardiomyocytes were preincubated for 15 min in the absence [control (C)] or presence of Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK) inhibitor STO-609 (5 \(\mu\)M) or CaMKII-specific inhibitor KN93 (5 \(\mu\)M) and then further treated with thapsigargin (Tpg) or A23187 (A23) for an additional 15 min. B: cardiomyocytes were incubated for 15 min in the absence (C) or presence of oligomycin (Oli; 5 \(\mu\)M) or 5-amino-1-\(\beta\)-D-ribofuranosyl-imidazole-4-carboxamide (AICAR; 1.5 mM); 4-Hz electric field stimulation was applied during the last 7 min of overall incubations. At the end of the incubations, cells were lysed, and phosphorylation of CaMKI and CaMKII was assessed by Western blotting. Caveolin-3 (Cav3) was used as a loading control. Values are displayed as means \(\pm\) SE (n = 5). *Vs. control; #vs. A23187 (P < 0.05).
concomitantly decreased content of GLUT4 (by 40, 50, and 25%, respectively) and of CD36 (by 50, 70, and 65%, respectively) from the low-density microsomal fraction that represents intracellular membrane compartments (Fig. 4B). Finally, immunodetection and microscopical visualization of CD36 in intact cardiomyocytes clearly demonstrated increased cell surface staining upon treatment with each stimulus (Fig. 4C).

In conclusion, just as with oligomycin, the two applied Ca\(^{2+}/H\) -raising agonists induce translocation of both GLUT4 and CD36 from intracellular membrane compartments to the sarcolemma.

**Effects of Ca\(^{2+}/H\)-Elevating Stimuli on Substrate Uptake Into Cardiomyocytes**

Based on the observation that thapsigargin and/or A23187 induce GLUT4 and CD36 translocation, we studied whether transporter membrane localization results in increased glucose and LCFA uptake rates, respectively. In agreement with Fig. 3, A and B, oligomycin potently induced AMPK-Thr\(^{172}\) and ACC-Ser\(^{97}\) phosphorylation and also phosphorylation of AS160, another AMPK substrate (Fig. 5A), and markedly enhanced deoxyglucose and palmitate uptake into cardiomyocytes (Fig. 5B). However, thapsigargin and A23187 were without any effect on phosphorylation of AMPK and its substrates ACC and AS160 and also did not affect deoxyglucose and palmitate uptake (Fig. 5, A and B). Together, these findings indicate that AMPK activation is not involved in Ca\(^{2+}/H\) -induced GLUT4 and CD36 translocation and that Ca\(^{2+}/H\)-induced translocation of both transporters as such is insufficient for increased glucose and/or LCFA uptake.

Apparently, Ca\(^{2+}/H\)-induced GLUT4 and CD36 translocation did not effectuate into increased uptake. However, it is still possible that coactivation of AMPK might be required to reveal a stimulatory action of the [Ca\(^{2+}/H\)]-raising agonists on glucose and LCFA uptake. For this purpose, we studied the effects of cotreatment of Ca\(^{2+}/H\) signaling activators with AMPK activators on AMPK signaling and on deoxyglucose and palmitate uptake. Thapsigargin and A23198, each unable to stimulate AMPK signaling by themselves, were also unsuccessful in further stimulating AMPK-Thr\(^{172}\), ACC-Ser\(^{97}\), and AS160-Thr\(^{642}\) phosphorylation in oligomycin-treated cardiomyocytes (Fig. 5A). However, remarkably, thapsigargin and A23187, each unable to stimulate substrate uptake by themselves, enhanced deoxyglucose uptake significantly in the presence of oligomycin cotreatment (1.4- and 1.3-fold, respectively) and palmitate uptake in the presence of either oligomycin cotreatment (each 1.3-fold) (Fig. 5B).
Fig. 4. Effects of [Ca\textsuperscript{2+}]-elevating agents on glucose transporter 4 (GLUT4) and CD36 translocation. Cardiomyocytes were preincubated for 15 min in the absence (C) or presence of Tpg (5 \mu M), A23 (5 \mu M), or Oli (5 \mu M, positive control). In selected experiments, cardiomyocytes were pretreated with STO-609 (5 \mu M) or KN93 (5 \mu M) for 15 min prior to addition of the [Ca\textsuperscript{2+}]-elevating stimuli. Following these treatments, cardiomyocytes were used for 3 different assays to detect transporter translocation. A: biotinylation assay; GLUT4 and CD36 were detected by Western blotting prior to biotinylation (total) and after biotinylation (surface). Signals were quantified using Quantity-One software (n = 5). Representative blots are shown. B: fractionation method; in the collected plasma membrane and low-density microsomal fractions, GLUT4 and CD36 were detected by Western blotting and quantified (n = 5). Representative blots are shown. C: 2-photon microscopy; CD36 was detected upon FITC labeling. Quantification of the CD36 signal was performed using ImageJ software (n = 5). Representative microscopic images are shown. Values are displayed as means \pm SE. *Vs. C; #vs. A23 (P < 0.05).
Effects of AMPK Inhibitors on Substrate Uptake Into Cardiomyocytes Treated With Ca\textsuperscript{2+} and AMPK Activators

To determine whether A23187-stimulated substrate uptake in the presence of AMPK coactivation was dependent on this AMPK coactivation and not due to off-target actions of the used AMPK activators, we incubated cardiomyocytes with established AMPK inhibitors, i.e., compound C and Ara-A. In the absence of these inhibitors, AICAR stimulated palmitate uptake 1.3-fold, and A23187 further stimulated palmitate uptake 1.3-fold, bringing the combined activation to 1.6-fold (Fig. 6\textsuperscript{A}). As expected, each of these AMPK inhibitors successfully inhibited AICAR-induced AMPK-Thr172 and ACC-Ser97 phosphorylation (Fig. 6\textsuperscript{B}). Although compound C and Ara-A did not alter basal palmitate uptake, each agent completely inhibited AICAR-induced palmitate uptake (Fig. 6\textsuperscript{A}). Furthermore, each agent completely inhibited A23187-stimulated palmitate uptake in the presence of AICAR co-treatment (Fig. 6\textsuperscript{A}).

In conclusion, the ability of A23187 to increase deoxyglucose and palmitate uptake into cardiomyocytes in the presence of AMPK activators indicates that Ca\textsuperscript{2+}-induced transporter translocation requires AMPK coactivation for effectuating such translocation into enhanced substrate uptake.

DISCUSSION

The aim of this study was to investigate the interrelation between [Ca\textsuperscript{2+}], CaMKK\textbeta/CaMks, and AMPK in the short-term regulation of substrate uptake into the heart. This short-term regulation is mediated by translocation of GLUT4 and CD36 to the cell surface. The major novel findings of this study are as follows: 1) Ca\textsuperscript{2+} signaling is not involved either in AMPK activation or in AMPK-mediated substrate uptake; 2) activation of Ca\textsuperscript{2+} signaling, similarly to AMPK signaling, stimulates translocation of GLUT4 and CD36 from endosomes to the sarcolemma; 3) in contrast, activation of Ca\textsuperscript{2+} signaling does not stimulate glucose or LCFA uptake, whereas AMPK signaling does; and finally, 4) Ca\textsuperscript{2+}-induced translocation of GLUT4 and/or CD36 can result in enhanced substrate uptake when accompanied with AMPK activation.

Together, these findings propose that Ca\textsuperscript{2+}-induced transporter translocation may help to recruit an additional quantity of substrate transporters to the sarcolemma to have these available to respond rapidly to conditions that require supraphysiological energy demands.

Ca\textsuperscript{2+} Signaling is Not Involved in Either AMPK Activation or AMPK-Mediated Substrate Uptake

In skeletal muscle, activation of Ca\textsuperscript{2+} signaling induces AMPK activation, and CaMKK\textbeta appears to be involved in contraction-induced AMPK activation (2, 28). In contrast, in our cardiomyocyte studies the [Ca\textsuperscript{2+}]-increasing stimuli A23187 and thapsigargin did not alter AMPK activation. Moreover, neither STO-609 nor KN93 affected contraction-induced AMPK activation or AMPK activation by AICAR or oligomycin, thus excluding a role for CaMKK\textbeta or CaMKII in the regulation of AMPK signaling in the heart. The inability of CaMKII to activate AMPK in cardiomyocytes is in agreement with previous work in skeletal muscle (48). However, the lack...
of involvement of CaMKKβ in AMPK activation in cardiomyocytes points toward differences in the regulation of AMPK between heart and skeletal muscle.

With respect to the regulation of substrate uptake, we observed that in cardiomyocytes AMPK-activating stimuli (including contraction) enhance GLUT4 and CD36 translocation, as well as glucose and LCFA uptake, in agreement with our previous studies (9, 21). The inability of STO-609 or KN93 to inhibit AMPK-mediated substrate uptake matches the inability of both inhibitors to affect AMPK signaling and excludes the involvement of CaMKKβ or CaMKII herein. These findings are in line with our previous observations in cardiomyocytes from LKB1-knockout mice, in which AMPK-mediated increases in glucose and LCFA uptake were lost entirely (21), and therefore, they provide further evidence that LKB1, and not CaMKKβ, is the AMPK-activating kinase involved in the contraction-induced regulation of cardiac substrate uptake.

**Activation of Ca²⁺ Signaling Stimulates Translocation of GLUT4 and CD36**

Although the effect of [Ca²⁺]i-increasing stimuli on glucose transport in skeletal muscle has been a topic of several studies (28, 46, 48), only few studies have shown GLUT4 translocation upon treatment with [Ca²⁺]i-increasing stimuli (e.g., see Ref. 2). To our knowledge, no studies have shown that an elevation in [Ca²⁺]i, induces GLUT4 translocation in cardiomyocytes. The present study fills this gap, because cell surface biotinylation as well as subcellular fractionation reveal that both A23187 and thapsigargin induce GLUT4 translocation. Reports on a relationship between Ca²⁺ signaling and CD36 translocation are limited to observations that caffeine induces CD36 translocation in skeletal muscle (2, 30), whereas no studies have been undertaken with respect to the heart. However, caffeine stimulates both Ca²⁺ signaling and AMPK signaling simultaneously, and therefore, this agent is not suited to study the effects of Ca²⁺ signaling separated from AMPK signaling. Each of the methods applied by us to assess CD36 translocation, including two-photon microscopy, clearly indicated enhanced CD36 translocation in response to A23187 and thapsigargin. The inability of these agents to activate AMPK indicates that Ca²⁺-induced CD36 translocation occur independently from AMPK signaling. Furthermore, both Ca²⁺-induced GLUT4 translocation and CD36 translocation are sensitive to inhibition by STO-609 and KN93, pointing to the involvement of CaMKKβ/CaMKs.

A positive control in our translocation studies was the translocation of both GLUT4 and CD36 in response to AMPK activation by oligomycin, in agreement with previous studies.
AMPK-mediated transporter translocation has been found to be dependent on phosphorylation-mediated inhibition of AS160 (18, 39), and in agreement, oligomycin treatment resulted in AS160 phosphorylation (Fig. 5A). Surprisingly, activation of Ca\(^{2+}\) signaling does not lead to phosphorylation of AS160, suggesting that AS160 does not regulate Ca\(^{2+}\)-induced transporter translocation. Perhaps Rab GTPase-activating proteins other than AS160 (i.e., its homologue TBC1D1) are involved herein.

Besides the fact that the cell surface biotinylation experiments provide evidence for Ca\(^{2+}\)-induced GLUT4 and CD36 translocation, these experiments also indicate that both transporters have properly arrived at the cell surface and have access to the extracellular milieu. This indicates that Ca\(^{2+}\) signaling is involved not only in the vesicle-mediated GLUT4 activation and CD36 translocation process but also in the subsequent fusion of the GLUT4- and CD36-containing vesicles with the plasma membrane.

**Activation of Ca\(^{2+}\) Signaling is Not Sufficient to Stimulate Glucose and LCFA Uptake**

In contrast to the parallel increases in AMPK-mediated substrate transporter translocation and AMPK-mediated substrate uptake, Ca\(^{2+}\)-induced GLUT4 and CD36 translocation was not accompanied by increases in glucose and LCFA uptake. A likely explanation for these findings is that substrate transporter translocation (including subsequent fusion with the plasma membrane) is necessary but not sufficient for a given stimulus to increase substrate uptake. Hence, once they arrive at the cell surface, substrate transporters may need an additional activation step to become fully functional in substrate uptake. In the case of AMPK signaling, this signaling route might then be involved in both transporter translocation and cell surface activation of the transporters. In contrast, Ca\(^{2+}\) signaling appears to be involved only in the first step, the translocation, and ineffective in further cell surface activation. Evidence for such a two-step process in the stimulation of substrate transport has been reported previously in adipocytes as the inability of phosphatidylinositol-3,4,5-trisphosphate (PIP\(_3\)) to stimulate glucose uptake into these cells despite a successful translocation of GLUT4 to the plasma membrane (41). These latter findings indicate that PIP\(_3\) [the main product of insulin-stimulated phosphatidylinositol-3 kinase (PI3K) activation] is involved in insulin-stimulated GLUT4 translocation, but the GLUT4 activation step required for insulin-stimulated glucose uptake is mediated by another, perhaps PI3K-independent, factor.

Similarly, in AMPK-mediated substrate uptake, the cell surface activation step would require different downstream mechanisms of AMPK compared with the translocation step. Specifically, whereas phosphorylation of AS160 (or TBC1D1) by AMPK is involved in the regulation of GLUT4 and CD36 translocation (29, 39), we do not necessarily expect AS160/TBC1D1 to be involved in cell surface transporter activation at the cell surface since the Rab GTPase activity of both proteins is tightly connected to vesicular trafficking (13).

We can only speculate about the mechanism of AMPK-mediated transporter activation at the cell surface. This activation step might involve a signaling-induced phosphorylation of each of the transporters individually. However, evidence for phosphorylation of GLUT4 or of CD36 to regulate transport activity is scarce. For CD36, merely ectophosphorylation was found to be dependent on phosphorylation-mediated inhibition of AS160 (18, 39), and in agreement, oligomycin treatment resulted in AS160 phosphorylation (Fig. 5A). Surprisingly, activation of Ca\(^{2+}\) signaling does not lead to phosphorylation of AS160, suggesting that AS160 does not regulate Ca\(^{2+}\)-induced transporter translocation. Perhaps Rab GTPase-activating proteins other than AS160 (i.e., its homologue TBC1D1) are involved herein.

**Fig. 7. Putative mechanism of the synergistic effect of Ca\(^{2+}\) and AMPK signaling on cardiac glucose and fatty acid uptake.** Contraction as induced by an action potential (AP) leads to activation of AMPK. Both contraction-induced AMPK activation and a supraphysiological influx of Ca\(^{2+}\) into cardiomyocytes (e.g., induced by A23187) trigger translocation of GLUT4 and CD36 from separate AMPK-responsive and Ca\(^{2+}\) signaling-responsive endosomal subcompartments to the sarcolemma. AS160 is involved in AMPK-mediated GLUT4/CD36 translocation but not in Ca\(^{2+}\)-induced transporter translocation. Both transporters arrive at the sarcolemma in an inactive state (depicted by a light gray fill color). Then, both transporters need an activation step [possibly lateral movement through the bilayer towards a specific membrane domain (depicted as a black membrane area)] to become functional in substrate uptake (depicted by a dark gray fill color). This latter step is dependent on AMPK activation. Hence, whereas AMPK-recruited transporters do not require additional signaling pathways to effectuate translocation into enhanced substrate uptake, Ca\(^{2+}\)-recruited transporters depend on additional AMPK activation to become functional.
Combined Activation of Ca²⁺ Signaling and AMPK Signaling Synergistically Stimulates Glucose and LCFA Uptake in an AMPK-Sensitive Manner

One major observation in this study is that activation of Ca²⁺ signaling, by itself unsuccessful in stimulating glucose and LCFA uptake into cardiomyocytes, substantially stimulated cardiac substrate uptake upon coactivation of AMPK signaling. Hence, Ca²⁺ signaling is dependent on separate coactivation of AMPK signaling to stimulate cardiac substrate uptake (illustrated in Fig. 7). In view of the ability of Ca²⁺ signaling to induce both GLUT4 and CD36 to translocate to the sarcolemma, once they have arrived at the cell surface, both transporters might undergo subsequent activation by AMPK signaling. The observation that pharmacological AMPK inhibitors entirely block substrate uptake induced by combined activation of Ca²⁺ and AMPK signaling provides support that activation of Ca²⁺-recruited transporters is indeed due to AMPK and not to an off-target effect of these stimuli.

The findings of this study point toward a gap in our knowledge of the role of Ca²⁺ and CaMKs on contraction stimulation of cardiac substrate uptake. Our findings revealed that upon an exaggerated rise in [Ca²⁺], by pharmacological agents, activation of CaMKs will induce both GLUT4 and CD36 translocation. However, contraction stimulation did not activate Ca²⁺ signaling, at least not within the observation time (several minutes). Perhaps Ca²⁺-induced/CaMK-mediated transporter translocation might gain importance during pathological Ca²⁺ overload conditions (17), as mimicked by thapsigargin or A23187 treatment. Under such pathological conditions, AMPK activity would not be expected to be impaired and thus amply sufficient to mediate cell surface transporter activation. Then, Ca²⁺-induced transporter translocation can be effectuated into increased substrate uptake. Together, the present findings point toward the hypothetical presence of separate GLUT4/CD36-containing subcompartments within the endosomes specifically responsive to a rise in [Ca²⁺], next to the AMPK-responsive storage compartments for these transporters. The recruitment of transporters from these Ca²⁺-responsive storage compartments might be restricted to emergency situations (like hypoxia and fight/flight response) to meet the excessive metabolic demands of the heart in such conditions.

Yet the basal activity of CaMKs in the heart or the changes in activation of CaMKs on a millisecond time scale are not yet known. Similarly to Ca²⁺ waves that activate CaMK signaling, AMPK activation status might also fluctuate on the millisecond time scale. Expectedly, there would be some delay to the CaMK signaling, because the increased energy demand (that is sensed by AMPK through changes in adenine nucleotide levels) evolves from the Ca²⁺-induced cardiomyocyte contraction. Thus, we could speculate about Ca²⁺/CaMK signaling preparing the cardiomyocyte for substrate uptake during contraction by inducing transporter translocation, which in the case of actual energy requirement is finalized by AMPK into substrate uptake to refill cellular energy. At present, it is not possible for us to determine CaMK and AMPK signaling changes occurring at such a rapid pace. In conclusion, we cannot exclude a possible millisecond action of CaMKs on transporter translocation under normal physiological conditions.

Finally, identification of the proteins functioning downstream of CaMKs in Ca²⁺-induced translocation of GLUT4 and CD36 might provide novel targets to influence cardiac substrate uptake.

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