INTRACELLULAR CALCIUM REGULATES AMP-ACTIVATED PROTEIN KINASE ACTIVITY IN AN OSCILLATION-DEPENDENT MANNER

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***Abstract*—Skeletal muscle calcium signaling is important for muscle contraction, as well as regulates many cellular processes. Calcium-regulated calmodulin dependent kinase kinase (CaMKK) has recently been identified as upstream regulator of AMP-activated protein kinase (AMPK), which is energy regulator in skeletal muscle. Although there is evidence that cytosolic calcium regulates AMPK through a series of pathways, the molecular mechanisms by which calcium regulates AMPK are poorly understood. The objective of this study is to understand the function of calcium oscillations on AMPK activity and define the specific calcium-regulated signaling molecules in this pathway. AMPK activity was increased by 2 folds in muscles from mice treated with AICAR (known AMPK activator). Administration of caffeine (calcium releasing agent) for 10 d decreased AICAR-induced AMPK activity to control level. This repressed AMPK activity was blocked by dantrolene, a ryanodine receptor stabilizer. Different calcium frequencies were simulated in C2C12 myotubes by alternating media containing caffeine and dantrolene. Changes in intracellular calcium levels were confirmed by fluorescent calcium indicator, Fura2. To define the function of calcium signaling, CaMKK or CaMK was knocked down. Low frequency calcium stimulations had a positive effect on AICAR-induced AMPK activity, whereas continuous high calcium level decreases AMPK activity suggesting a biphasic control of AMPK activity by calcium. Knock-down studies suggest the negative effects of chronic calcium levels on AMPK activity is partly mediated through the CaMK signaling cascade. These data show different cytosolic calcium waves elucite distinctly responses in muscle cells and suggest that inhibition of AMPK by calcium is mediated in part by the calcium-CaMK signaling cascade.**

***Index Terms*—AMPK, calcium, skeletal muscle, signal transduction.**

I. INTRODUCTION

Cytosolic calcium not only controls muscle contraction and relaxation, but also serves as a second messenger to trigger pathways that regulate muscle function, plasticity, gene expression, and energy metabolism. Given that cytosolic calcium levels are dynamic and reflect muscle function, effective signaling may result from the frequency of calcium oscillations rather than by the level of cytosolic calicum (Chin, 2005). Calcium signals are propagated as waves or oscillations and act as an intracellular messenger by activating calcium responsive proteins, such as protein kinase C, calcium-calmodulin dependent kinase (CaMK), and protein phosphatase 2B (calcineurin). However, there is evidence that calcineurin is not involved in phenotypic modifications of skeletal muscle with repeated contraction (Garcia-Roves *et al.*, 2006). Increases in cytosolic calcium during contraction also reduce ATP:AMP and phosphocreatine:creatine ratios and these changes, in turn stimulates AMP-activated protein kinase (AMPK)(Hardie *et al.*, 2003).

AMPK senses energy status in skeletal muscle and modifies its inherent energy processes (Hardie *et al.*, 2003). In response to stimuli that decrease energy charge, as indicated by increase in AMP:ATP ratio, AMPK is activated via phosphorylation by upstream kinases. Although the tumor suppressor LKB1 is considered the major AMPK kinase, it is not likely a direct regulator of AMPK activity as AMPK can be activated in LKB1 deficient cells as well as LKB1 is constitutively active (Lizcano *et al.*, 2004). AMP promotes phosphorylation also by protecting AMPK from dephosphorylation, which is likely catalyzed by protein phosphatases (Winder, 2001). Once activated, AMPK regulates various downstream pathways to preserve cellular ATP levels. To that end, AMPK turns on catabolic processes and shuts off energy consuming anabolic pathways.

Calcium signals modulate skeletal muscle adaptation via a CaMK signaling cascade. Among the multifunctional CaMKs, CaMKKα or CaMKII is a strong candidate for mediating calcium signaling in skeletal muscle due to their muscle specific expression. There is conflicting evidence whether calcium signals positively or negatively regulate AMPK activity. Recent studies have shown that an increase in cytosolic calcium activates AMPK via a CaMKK-dependent signaling cascade; AMPK is activated by calcium ionophores in LKB1 deficient cells, and this activation is blocked by CaMKK inhibitor STO609 or isoform-specific small interfering RNAs (siRNA) in vitro (Hurley *et al.*, 2005) and CaMKKα has been implicated in increasing AMPK activity in response to α-lipoic acid (Shen *et al.*, 2007). However, our previous study have shown that sustained increases in cytosolic calcium caused by caffeine or calcium ionophores either inhibited or had no effect on AMPK phosphorylation (Park *et al.*, 2009). These results suggest that calcium modulates AMPK activity and that this regulatory function is dependent on the amplitude and duration of calcium oscillation.

Given the important roles of AMPK in the regulation of whole body energy metabolism, it is plausible that cytosolic calcium is a component the AMPK regulatory pathway in skeletal muscle. Although there is evidence that cytosolic calcium regulates AMPK, the molecular mechanisms by which calcium transients regulate AMPK are not fully understood. Thus, the purpose of this study is to understand the function of calcium oscillations on AMPK activity and define the specific calcium-regulated signaling molecules in this pathway.

II. MATERIALS AND METHODS

Animals

Male C57/B6 mice aged 6 wks were treated with saline (control), AICAR (0.1M), caffeine, AICAR+caffeine, dantrolene (20mg/kg body weight), or in combination (n=4 per group). For the caffeine-treated group, mice were allowed to drink caffeinated water (0.3 g/L in tap water). At day 10, longissimus dorsi (LD) muscles were removed and snap-frozen in liquid nitrogen.

AMPK assay

AMPK activity was measured using SAMS peptide as previously described (DAVIES *et al.*, 1989). Briefly, powdered LD muscles were homogenized with lysis buffer containing 0.25 M mannitol, 0.05 M Tris-HCL, 1 mM EDTA, EGTA, DTT, 5 mM sodium pyrophosphate, 50 mM NaF, 2 mM PMSF, and leupeptin and centrifuged at 13,000 g for 5 min at 4 ˚C, then 10 µl of supernatant was incubated for 10 min at 37 ˚C in 40 mM HEPES, 0.2 mM SAMS peptide (HMRSAMSGLHLVKRR; GenScript, Piscatawasy, NJ), 0.2 mM AMP, 80 mM NaCl, 8% w/v glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM $MgCl_2$, and 0.2 mM ATP + 2 µCi [^{32}P] ATP, pH 7.0. An aliquot was spotted on a Whatman P81 paper and radioactivity was quantified. Activity was expressed as the phosphorylation of mM peptide/min/g of muscle.

Cell culture

C2C12 myotubes were seeded in DMEM supplemented with 10% (v/v) FBS and antibiotics. At 80% confluence, cells were switched to a differentiation media (DM) containing 4% horse serum. Fully fused myotubes were treated with 2 mM AICAR, 3 mM caffeine, 1 mM dantrolene, 10 µM KN62, 10 µM KN93, 10 µg/ml STO609, or in combinations. Ten h after treatment, C2C12 myotubes were rinsed with PBS and homogenized in ice cold RIPA buffer with phosphatase inhibitor cocktail (Sigma, St. Louis, MO).

Intracellular Calcium measurement and calcium oscillation generation

Intracellular calcium was visualized by using Fura2, a fluorescent calcium dye. C2C12 myotubes grown on glass cover slips were loaded with 5 µM Fura2 for 40 min. After incubation, C2C12 myotubes were rinsed with pre-warmed PBS, and intracellular calcium concentration was measured using Eclipse Ti microscope (Nikon Corp., Tokyo, Japan) with a Xenon lamp and smart shutter (Sutter Instrument Co., Navato, CA, USA). Fluorescence was recorded by calculating the ratio of Fura2 fluorescence at 510 nm excited by ultraviolet light at 340 (calcium bound) and 380 (calcium unbound) nm. To induce the release of calcium from the sarcoplasmic reticulum (SR), 3 mM caffeine was added to the culture. To desensitize the SR, and thereby decrease cytosolic calcium level, 1 mM dantrolene was added. For prolonged high calcium level, C2C12 myotubes were continuously incubated in DM containing AICAR, caffeine, dantrolene, or in combination for 10 h. To create calcium oscillations, C2C12 myotubes were incubated with caffeine containing DM for 20 min, briefly washed with pre-warmed PBS, and incubated with dantrolene containing DM for 3 min. This caffeine-dantrolene media switching cycle was continued for 10 h.

RNA interference

Small interference RNA (siRNA) was used to knock-down CaMKK expression. At 80% confluence, C2C12 cells were transfected with scrambled (control) or CaMKKα-specific siRNA. After 48 h, media were switched to DM. After 4 d, fully differentiated myotubes were treated with 2 mM AICAR, 3 mM caffeine, and in combination for 10 h.

Western blotting

Thirty micrograms of protein were resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked, and immunoblotted with primary antibodies for 2 h. Secondary antibodies conjugated with horseradish peroxidase (1:1000) were applied for 1 h and bands were visualized using enhanced chemiluminescence (ECL; Amersham) and quantified using NIH ImageJ.

Statistics

Data are reported as mean ± s.e. and compared with Student's *t* test. Values were considered significant at the $P < 0.05$ level.

III. RESULTS AND DISCUSSION

Cytosolic calcium and AMPK are key factors in skeletal muscle energy metabolism and adaptation. However, the interaction between these signals is not well understood. The present study established a biphasic effect of calcium on AMPK activity. AMPK activity assessed by SAMS peptide (Fig.1) was increased by AICAR treatment more than 50% compared to control mice ($p < 0.01$). Interestingly, caffeine treatment reduced AICAR-induced AMPK activity to control levels. Administration of dantrolene abolished the negative effect of caffeine on AMPK activity. The negative involvement of calcium signals in AMPK activity is novel.

Cytosolic calcium acts as a second messenger and signals to downstream molecules by its oscillation in the form of calcium spikes (Bootman *et al.*, 2001). We hypothesized that the functional role of calcium on AMPK activity is dependent on its oscillation. To test this, we induced varying calcium oscillations using caffeine and dantrolene. Cytosolic calcium level in C2C12 myotubes was monitored by Fura2. Administration of caffeine for 10 h maintained a 2-fold higher cytosolic calcium level compared to control group (data not shown). When caffeine was added into the control myotubes, calcium ratio 340/380 was increased from 0.1 to 0.17 (+caffeine; Fig. 2). When dantrolene was added (+ dantrolene), 340/380 ratio returned to basal levels. When caffeine was added back into the media, a second calcium spike was observed and was again eliminated by the subsequent addition of dantrolene. This confirms that, in our hands, dantrolene media could be alternated with caffeine to create calcium oscillations in muscle cell culture.

To further examine the effect of calcium oscillations on the regulation of AMPK activity, we compared sustained high calcium versus concomitant calcium spikes. AICAR treatment increased the phosphorylation of AMPK and its downstream signal, ACC. Sustained caffeine treatment had no effect on AMPK or ACC phosphorylation. Similar to muscles from mice treated with AICAR and caffeine shown in Figure 1, continuous caffeine treatment decreased the AICAR-induced AMPK and ACC phosphorylation to control level in C2C12 myotubes (AIC + caf; Fig. 3). However, cells treated with intermittent calcium spikes (switch) displayed a positive effect of caffeine on AICAR-induced AMPK activity (Fig. 3). These data support our hypothesis and provide a biphasic effect of calcium in regulating AMPK activity.

Several lines of evidence suggest that CaMKK acts upstream of calcium-regulated signaling molecules (Soderling, 1999) and also mediates calcium-induced activation of AMPK in mammalian cells (Hurley *et al.*, 2005). Isoforms of CaMKKs, CaMKKα and CaMKKβ, are similar in their amino acid sequence, but CaMKKβ is not expressed in muscle (Witczak *et al.*, 2007). Furthermore, previous research indicates that CaMKII serves as a molecular decoder of information stored in the form of calcium oscillation frequencies (DeKoninck and Schulman, 1998). To further investigate the molecular mechanisms by which sustained high calcium negatively regulates AICAR-induced AMPK activity, we utilized inhibitors and siRNA that are specific for calcium-mediated signaling molecules, including CaMKKα and CaMKII. Both siRNA specific for CaMKKα, and the CaMKII inhibitor KN93, silenced CaMKKα expression and decreased phosphorylation of CaMKII, respectively (Fig. 4). However, the inhibition of CaMKKα did not fully rescue the inhibitory effect of chronic calcium on AMPK activity (data not shown). Rather, inhibition of CaMKII activity by KN93 treatment abolished the negative effect of calcium on AMPK activity and conserved AICAR-induced AMPK activity in the presence of caffeine (Fig. 5). These data support a functional role of CaMKII in the calcium-AMPK signaling cascade and imply that chronic high calcium decreases AICAR-induced AMPK activity via the CaMKII signaling molecule.

V. CONCLUSION

Calcium regulates AMPK activity in an oscillation frequency-dependent manner. Using both a mouse and cell culture model, we demonstrated that sustained calcium stimulation decreases AICAR-induced AMPK activity. By incorporating a cycling protocol for C2C12 myotubes that alternates between caffeine and dantrolene containing media, we created intermittent calcium spikes that, in contrast to the chronic model, had a positive effect on AICAR-induced AMPK activity. In addition, a specific CaMKII inhibitor eliminated the negative effect of caffeine on AMPK activity, supporting our hypothesis that regulation of AMPK activity by chronic calcium is partly achieved through the calcium-CaMKII signaling cascade.

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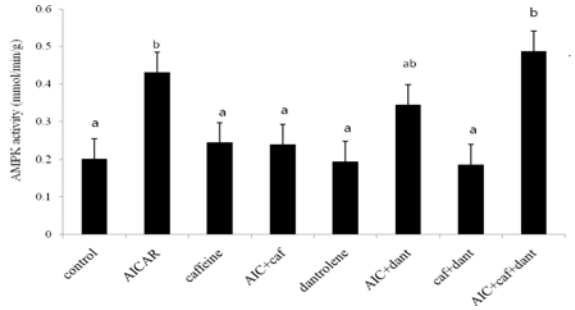

Figure 1. AMPK activity in longissimus dorsi muscles from saline, AICAR, caffeine, and dantrolene treated mice. AMPK activity (n=4 in each group) was measured by synthetic SAMS peptide and P^{32} incorporation. Different letters indicate significance ($P < 0.05$, n=4).

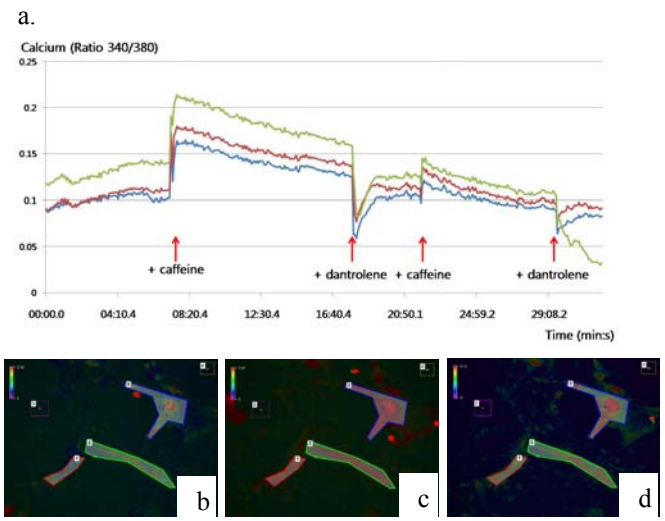

Figure 2. Cytosolic calcium concentration of C2C12 myotubes. Intracellular calcium was imaged by Fura2, a fluorescent calcium dye, as described in Materials and Methods. 2a, graphic representation of the integrated cytosolic calcium measured by 340/380 ratio (arrows indicate the addition of caffeine or dantrolene). 2b, 2c, and 2d are fluorescence images of the myotube in basal (b), caffeine treated (c), and dantrolene treated (d) myotubes.

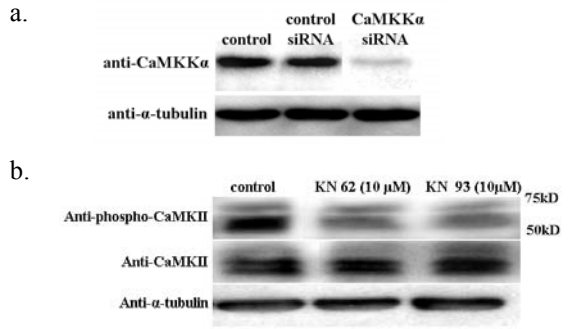

Figure 4. Knock-down of CaMKKα expression by siRNA specific for CaMKKα (a). Phosphorylation of CaMKII in the presence of CaMK inhibitors, KN62 and KN93 (b).

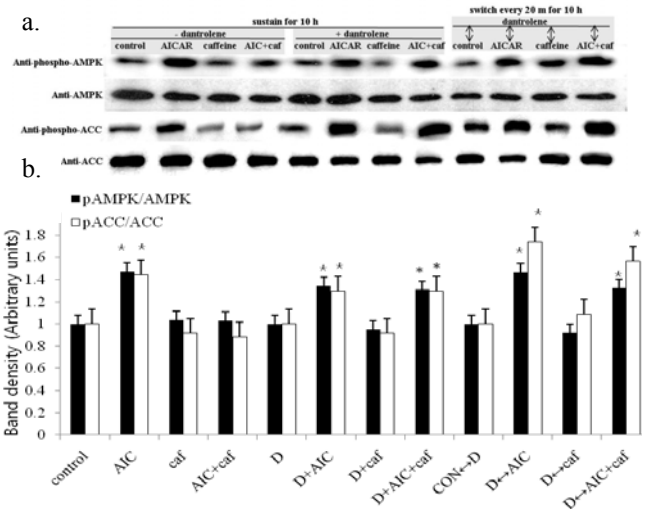

Figure 3. Phosphorylation of AMPK and ACC in C2C12 myotubes treated with AICAR (AIC), caffeine (caf), and dantrolene (D) for 10h. Sustained high calcium levels were maintained by continuous incubation of caffeine and intermittent calcium spikes were created by switching dantrolene containing media with caffeine containing media (D↔caf). 3a, representative images and 3b, average band density of 3 independent western blots. * indicates significance ($P < 0.05$).

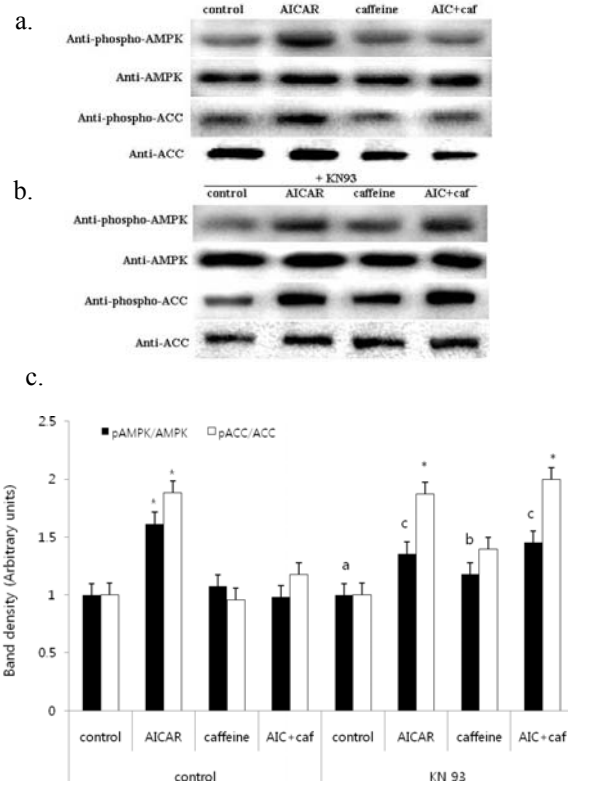

Figure 5. Phosphorylation of AMPK and ACC in C2C12 myotubes treated with vehicle (control), AICAR, caffeine, and AICAR+caffeine without (a) or with KN93 (b). 3c, average band intensity of 3 independent western blots. * or different letters indicate significance ($P < 0.05$).