Original article

MOLECULAR METABOLISM

Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca^{2+} release

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ABSTRACT

Understanding how muscle contraction orchestrates insulin-independent muscle glucose transport may enable development of hyperglycemia-treating drugs. The prevailing concept implicates Ca^{2+} as a key feed forward regulator of glucose transport with secondary fine-tuning by metabolic feedback signals through proteins such as AMPK. Here, we demonstrate in incubated mouse muscle that Ca^{2+} release is neither sufficient nor strictly necessary to increase glucose transport. Rather, the glucose transport response is associated with metabolic feedback signals through AMPK, and mechanical stress-activated signals. Furthermore, artificial stimulation of AMPK combined with passive stretch of muscle is additive and sufficient to elicit the full contraction glucose transport response. These results suggest that ATP-turnover and mechanical stress feedback are sufficient to fully increase glucose transport during muscle contraction, and call for a major reconsideration of the established Ca^{2+} centric paradigm.

Keywords Exercise; Skeletal muscle; Ca^{2+}; AMPK; Stretch

1. INTRODUCTION

Muscle contraction during exercise is a more potent physiological stimulus of skeletal muscle glucose uptake than even maximal insulin [1] and a highly effective prophylactic against the hyperglycemia associated with the development of type 2 diabetes mellitus [2]. Despite these drug-target-wise attractive features, the molecular signalling mechanisms by which muscle contraction augments glucose transport are, compared to insulin, relatively understudied and remain poorly defined (for review see Ref. [3]).

The signals underlying increased glucose transport with contraction have long been speculated to include a feedforward signalling component, activated directly by depolarization-induced SR Ca^{2+} release through the ryanodine receptors. In addition, feedback signals are activated secondarily in response to Ca^{2+}-activated contraction to further fine-tune the glucose transport response, including probably activation of the metabolic stress responsive kinase 5′ AMP-activated protein kinase (AMPK) by ATP-turnover and mechanical stress-activated signals [4,5]. The belief that Ca^{2+} by itself is adequate to stimulate glucose transport is based to a major extent on seminal work by John Holloszy and co-workers where 3–4 mM caffeine was shown to increase SR Ca^{2+} release and glucose transport in incubated rat muscles without changes in muscle tension, ATP or Creatine phosphate [6]. In rats, caffeine-stimulation was sufficient to double glucose transport rate in slow-twitch oxidative soleus muscle and triple it in fast-twitch glycolytic epitrochlearis [7,8] without activating AMPK [7]. This suggested that Ca^{2+} *per se* could stimulate a substantial increase in muscle glucose transport. However, our group and others subsequently found 3–4 mM caffeine increased AMPK activation and nucleotide-turnover in muscles from mice and rats [9–11], presumably due to the considerable energy-demand posed by sarco/endoplasmatic reticulum Ca^{2+} ATPase (SERCA)-dependent Ca^{2+} reuptake [12]. Furthermore, caffeine-stimulated glucose transport was potently inhibited in muscles from muscle-specific dominant-negative kinase-dead α2 AMPK mice [10,13], suggesting that the caffeine-response largely depends on ATP turnover-mediated activation of AMPK rather than on Ca^{2+} as such.

To clarify the relative sufficiency and necessity of SR Ca^{2+} vs. feed-back signals to contraction-stimulated glucose transport, we presently combined contractile myosin blockers, AMPK transgenic mice, Ca^{2+}

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ATPase inhibitors and electrical stimulated contraction, in incubated mouse muscles, a classical model system allowing cell culture-like manipulations and full environmental control of fully differentiated, contraction-competent, striated muscle.

2. MATERIALS AND METHODS

2.1. Antibodies, reagents and immunoblotting

All antibodies and reagents used were commercially available. Details on antibodies used and immunoblotting specifics are included in Supplemental Experimental Procedures.

2.2. *Ex vivo* muscle incubation

Soleus and EDL muscles from anaesthetized female C57BL/6 wildtype and muscle-specific KD AMPK overexpressing [4] mice were incubated in continuously gassed (95% O_2/5% CO_2) modified Krebs–Ringer–Henseleit-buffer at 30 °C. After 10–15 min rest, buffer containing inhibitors was added for 1 h, followed by stimulation with CPA, insulin, electrical stimulation, AICAR or passive stretch as described in the figure legends and in the Supplemental Experimental Procedures.

2.3. Cytosolic Ca^{2+} measurements

Changes in Ca^{2+} concentration were measured as Fluo-3 fluorescence in confluent 3 h serum-starved L6 myotubes. See Supplemental Experimental Procedures for details.

2.4. AMPK activity

Immunoprecipitated AMPK trimer activity was measured *in vitro* using ^{32}P-labelled ATP incorporation into a substrate peptide. See Supplemental Experimental Procedures for details.

2.5. Statistics

The data were analysed using *T*-test or one-way, two-way or two-way repeated measures ANOVA with Tukey's post hoc test. The significance level was set at $p < 0.05$.

3. RESULTS

3.1. Myosin ATPase blockade with BTS and blebbistatin blocks contraction but not SR Ca^{2+} release

Ex vivo incubated mouse slow-twitch oxidative soleus and fast-twitch glycolytic extensor digitorum longus (EDL) muscles were stimulated with the SERCA-inhibitor cyclopiazonic acid (CPA) at increasing doses to inhibit Ca^{2+} reuptake, thus allowing a sustained Ca^{2+} leak from the sarco/endoplasmatic reticulum (SR). In soleus muscle, this caused a dose-dependent increase in the Ca^{2+}-regulated Thr57 phosphorylation of eEF2 by eEF2 kinase, a readout of SR Ca^{2+} release [14], in addition to increased phosphorylation of AMPK Thr172 (Figure 1A). This pattern is reminiscent of the increase in eEF2 and AMPK phosphorylation seen in soleus stimulated with 4–6 mM caffeine (Figure S1A). Glucose transport was elevated significantly above baseline using 50 μM CPA (Figure 1B). No effect of CPA was observed in mouse EDL (Figure 1A), probably due to the known differences in Ca^{2+} handling proteins between type II fibres compared to type I fibres [15]. Pretreatment with the SR Ca^{2+} channel blocker dantrolene prevented both the CPA-stimulated eEF2 and AMPK phosphorylations (Figure 1C) and directly measured Ca^{2+} release in L6 myotubes (Figure 1D), showing their dependence on SR Ca^{2+} release. The fast and slow contractile myosin heavy chain type II ATPase inhibitors, BTS and blebbistatin (Bleb) did not affect Ca^{2+} release (Figure 1D) consistent with previous reports [16–18].

The CPA-induced rise in cytosolic Ca^{2+} leak elicited a sustained submaximal muscle contraction (Figure 1E). Inhibition of contractile function by BTS + Bleb blocked tension development (Figure 1E + dose–response curves in Figure S1B), caused a general increase in the creatine phosphate/creatine (CrP/Cr)-ratio (Figure 1F) and markedly reduced CPA-induced metabolic stress measured as lactate concentration (Figure 1G).

3.2. Myosin ATPase blockade does not affect insulin or AICAR-stimulated signalling or glucose transport

We were initially concerned that Bleb in particular might have off-target effects since MyoII has been implicated in insulin-stimulated GLUT4 translocation and glucose transport in adipocytes [19]. However, neither insulin nor AICAR-stimulated signalling (Figure 2A+C) or glucose transport (Figure 2B+D) were inhibited by BTS + Bleb, suggesting that the effects of myosin ATPase inhibition are specific to contraction.

3.3. Reduced CPA-stimulated glucose transport with BTS + blebbistatin correlates with lower AMPK and mechanical stress-, but not SR Ca^{2+} dependent, signalling

Myosin ATPase inhibition in soleus muscle potently dampened the CPA-induced phosphorylation of AMPK and p38 MAPK, (Figure 2A) activated by ATP-turnover [4] and mechanical stress [20], respectively. SR Ca^{2+} dependent phosphorylation of eEF2 was unaffected (Figure 3A). BTS + Bleb significantly inhibited CPA-stimulated α2 AMPK activity and had a qualitatively similar effect on α1 AMPK activity (Figure S1C). Myosin ATPase blockade potently reduced CPA-stimulated glucose transport (Figure 3).

Next, we asked if the residual CPA-stimulated glucose transport-response in BTS + Bleb-treated soleus muscles required AMPK signalling. As expected, our signalling readouts including the phosphorylation of AMPK by upstream kinases on Thr172 were not affected by muscle-specific overexpression of kinase-dead α2 (KD) AMPK (Figure 3B). CPA-stimulated glucose transport was unaffected by muscle-specific overexpression of KD AMPK (Figure 3E).

Similar to the findings with BTS + Bleb in Figure 3A, combining BTS + Bleb with KD AMPK inhibition only reduced AMPK Thr172 and p38 MAPK Thr180/Tyr182 phosphorylation, but did not affect eEF2 Thr57 phosphorylation (Figure 3C). Interestingly, despite the normal SR Ca^{2+} dependent signalling, the residual glucose transport-response in the presence of BTS + Bleb was completely abolished in the presence of KD AMPK (Figure 3F). No effect of CPA or BTS + Bleb was found for total protein expression (Figure S1D). Overall, these data show that CPA-induced contraction-stimulated glucose transport can be completely prevented despite normal SR Ca^{2+} release. This strongly implies that SR Ca^{2+} in itself is insufficient to stimulate glucose transport. Rather, this appears to require input from AMPK and likely mechanical stress-signalling.

3.4. Contraction-stimulated glucose transport is related to metabolic and mechanical stress but not Ca^{2+} transients

A sustained increase in cytosolic Ca^{2+} by CPA is quite dissimilar from the transient Ca^{2+} spikes elicited by physiological muscle excitation–contraction coupling. Therefore, we also investigated the effect of contractile inhibition on the glucose transport-response elicited by intermittent electrical field stimulation of muscle contraction. Given that two previous reports saw widely different effect of BTS on the muscle glucose transport-response in rodent fast-twitch muscles (~50% reduction vs. no effect) [21,22], these previously published stimulation protocols were included in our

