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Effect of stimulation frequency on contraction-induced glucose transport in rat skeletal muscle

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Ihlemann, Jacob, Thorkil Ploug, Ylva Hellsten, and Henrik Galbo. Effect of stimulation frequency on contraction-induced glucose transport in rat skeletal muscle. Am J Physiol Endocrinol Metab 279: E862–E867, 2000.—Previous studies have indicated that frequency of stimulation is a major determinant of glucose transport in contracting muscle. We have now studied whether this is so also when total force development or metabolic rate is kept constant. Incubated soleus muscles were electrically stimulated to perform repeated tetanic contractions at four different frequencies (0.25, 0.5, 1, and 2 Hz) for 10 min. Resting length was adjusted to achieve identical total force development or metabolic rate (glycogen depletion and lactate accumulation). Overall, at constant total force development, glucose transport (2-deoxyglucose uptake) increased with stimulation frequency \( P < 0.05 \); basal: 25 ± 2, 0.25 Hz; 50 ± 4, 0.5 Hz; 50 ± 3, 1 Hz; 81 ± 5, 2 Hz; 79 ± 3 mmol·g\(^{-1}\)·min\(^{-1}\). However, glucose transport was identical \( P > 0.05 \) at the two lower (0.25 and 0.5 Hz) as well as at the two higher (1 and 2 Hz) frequencies. Glycogen decreased \( P < 0.05 \); basal: 19 ± 1, 0.25 Hz; 13 ± 1, 0.5 Hz; 12 ± 2, 1 Hz; 7 ± 1, 2 Hz; 7 ± 1 mmol/kg) and 5'AMP-activated protein kinase (AMPK) activity increased \( P < 0.05 \); basal: 1.7 ± 0.4, 0.25 Hz; 32.4 ± 7.0, 0.5 Hz; 36.5 ± 2.1, 1 Hz; 63.4 ± 8.0, 2 Hz; 67.0 ± 13.4 pmol·mg\(^{-1}\)·min\(^{-1}\) when glucose transport increased. Experiments with constant metabolic rate were carried out in soleus, flexor digitorum brevis, and epitrochlearis muscles. In all muscles, glucose transport was identical at 0.5 and 2 Hz \( P > 0.05 \); also, AMPK activity did not increase with stimulation frequency. In conclusion, muscle glucose transport increases with stimulation frequency but only in the face of energy depletion and increase in AMPK activity. This indicates that contraction-induced glucose transport is elicited by metabolic demands rather than by events occurring early during the excitation-contraction coupling.

5'-adenosine monophosphate-activated protein kinase; metabolism; exercise; GLUT-4; signal transduction

CONTRACTION and INSULIN both stimulate glucose transport in skeletal muscle; however, the two stimuli act via at least partially independent pathways (8, 13, 15–17) that are far from fully clarified. Two principal mechanisms exist by which contractions, as opposed to insulin, might regulate glucose transport. First, the depolarization of the plasma and T-tubule membranes preceding contractions might via second messengers, e.g., Ca\(^{2+}\) (23), stimulate translocation of the glucose transporter GLUT-4 from intracellular storage vesicles to the cell surface (4, 19). Second, glucose transport might be related to the strain put on the muscle or the work done by the muscle, and hence regulated by factors coupled to force development or metabolic rate. The first mechanism is in line with a previous study of frog sartorius muscle, from which it was concluded that the stimulation frequency, but not the workload, was of importance for the contraction-induced glucose transport (7). This view has been generally accepted for decades. However, in line with the second view, we have recently shown in rat soleus muscle that at a given stimulation frequency, glucose transport varies directly with total force development, metabolic rate, and 5'-AMP-activated protein kinase (AMPK) activity (9). Apparently, in rat soleus muscle, in contrast to frog sartorius muscle, contraction-stimulated glucose transport is not solely determined by stimulation frequency. In fact, an effect of stimulation frequency on glucose transport, dissociated from effects of mechanical performance and metabolism, remains to be seen in rat muscle. Such an effect would, however, agree with the finding that protein kinase C (PKC) and calmodulin inhibitors may impair contraction-stimulated glucose transport (8). Therefore, in the present study, we have further elucidated the relationship between stimulation frequency, total force development, and metabolism, on the one hand, and glucose transport in contracting rat soleus muscle on the other. The effect of stimulation frequency on glucose transport was studied by varying stimulation frequency while total force development or metabolism, as reflected by glycogen depletion and lactate accumulation, was kept constant. We used soleus muscles from young rats (65–75 g), which have a less uniform fiber-type composition than adult rats [52% type I, 33% type IIa, and 15% type IIc fibers (unpublished data) vs. 87% type I and 13% type IIa fibers (1)]. Nevertheless, to be sure that conclusions also applied to other muscles, the effect of varying...
stimulation frequency at constant metabolic rate was also studied in flexor digitorum brevis (FDB, 6% type I, 61% type IIa, 3% type IIb, and 30% type IIc [unpublished data]) and epitrochlearis (6% type I, 21% type IIa, 68% type IIb, and 5% type IIc [unpublished data]) muscles.

MATERIALS AND METHODS

Materials. 2-Deoxy-d-glucose (2-DG), glucose, bovine serum albumin fraction V (BSA), pyruvic acid, glutamic acid, trisma base, dithiothreitol (DTT), AMP, ADP, ATP, IMP, and sucrose were from Sigma. 2-Deoxy-d-[3H]glucose and [14C]sucrose were from Du Pont-NEN. Lactate dehydrogenase, lactate, glutamate-pyruvate-transaminase, adenosine-5-panthenoic acid, hexokinase, glucose-6-phosphate dehydrogenase, NADP, and NAD+ were from Boehringer.

Muscle incubation and stimulation. The experiments were approved by the Animal Research Committee of the Ministry of Justice and were in accordance with the animal experiment guidelines of research of the American Physiological Society. Fed male Wistar rats (65–75 g body wt) were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The rats were perfused through the left ventricle for 1 min (flow 20 ml/min) with Krebs-Henseleit bicarbonate-buffered medium containing 8 mM glucose, 1 mM pyruvic acid, and 0.2% BSA. The soleus, FDB, and epitrochlearis muscles were gently dissected free, with intact tendons at both ends, and incubated for 2 h (soleus) or 30 min (FDB and epitrochlearis) in perfusion medium in test tubes at 29°C. The medium was continuously gassed with 95% O2-5% CO2. Just before stimulation, the perfusion medium was replaced with glucose-free incubation medium: Krebs-Henseleit buffer containing 2 mM pyruvic acid and 0.2% BSA. Muscles were directly electrically stimulated to contract in vitro. A small clip was attached to each of the two tendons, and the muscle was vertically suspended in incubation medium with the upper end attached to a force transducer connected to a computer. Electrodes were placed at both ends of the muscle, and 10 min of repeated tetanic contractions were produced by stimulating with 25 V in 200-ms trains of 100 Hz, each impulse being 0.2 ms. Train frequency was either 0.25, 0.5, 1, or 2 Hz. The resting length yielding maximal force was used during stimulation at 0.25 Hz. When other frequencies were applied, resting length was adjusted, according to experience from preliminary experiments, to achieve either the same integrated force or the same glycogen depletion and lactate accumulation.

Measurement of total force development. The force development was recorded with software developed by the engineer of our department, H. P. Nissen. During every tetanus, 500 tension values of 8-bit resolution were recorded. The tension-time area under the curve for each tetanus was determined by adding the 500 values and subtracting resting tension. The tension-time areas for all tetani during the 10-min stimulation period were added and constituted the “total force development.” The tension-time areas were measured in arbitrary units. The total force development values were then normalized by dividing by the area produced by a 10-g weight (~0.1 N) hanging from the force transducer in a 200-ms period.

Measurement of 2-DG transport. 2-DG transport was measured as in Ref. 9. Briefly, immediately after contractions, muscle clips were removed, and the relaxed muscles were placed in test tubes for measurement of glucose transport. Transport was measured as 2-deoxy-d-[3H]glucose (2-[^3H]DG) uptake, with [14C]sucrose as extracellular marker. Isotopes and unlabeled sugars were added to the incubation medium to yield final concentrations of 0.43 μCi 2-[^3H]DG and 0.32 μCi [14C]sucrose per ml and 1 mM of both unlabeled 2-DG and sucrose. After 10 min of exposure to isotopes, muscles were briefly blotted on filter paper and immediately frozen in liquid nitrogen. Muscles were stored at –80°C until analyzed.

Measurement of metabolites and AMPK activity. Muscles were quickly removed from the incubation medium and, while still connected to the electrodes and still contracting, they were freeze-clamped with tongs cooled in liquid nitrogen and stored at –80°C until measurement of metabolite concentrations. Glycogen was determined by a hexokinase method after hydrolysis with HCl (11). Lactate and phosphocreatine (PCr) were determined by standard enzymatic fluorometric methods (14). Nucleotide concentrations were determined in neutralized perchloric acid extracts by reverse-phase HPLC (20). Separation was achieved by a 30-min gradient elution using a Hibar Lichrosphere 100 CH-18/2 (Merck) column (250 × 4 mm). The linear gradient program for the mobile phase was as follows: 0 min, 100% A; 0.1–4 min, 75% A; 4–17 min, 0% A; 17–22 min, 0–100% A. The composition of buffer A was 150 mM ammonium phosphate (pH 5.80). Buffer B also contained 150 mM ammonium phosphate, with 20% methanol and 2% acetonitrile added as organic modifiers (pH 5.45). Separation was achieved at room temperature with a flow of 0.8 ml/min. Detection was at 254 nm, and peaks were identified by comparison of retention times with commercially obtained compounds.

For AMPK activity determination, muscles were homogenized with a Polytron PT 3100 (Kinetica, Littau-Luzern, Switzerland) at maximum speed for 10–15 s in ~20 volumes of ice-cold buffer (in mM: 210 sucrose, 1 EDTA, 5 sodium pyrophosphate, 50 NaF, 1 DTT, 2 phenylmethylsulfonyl fluoride, and 50 HEPES, pH 7.4). The homogenate was centrifuged for 45 s at 15,000 g, and the supernatant was stored in aliquots for later determination of protein concentration and AMPK activity. Sample protein was determined by the Coomassie Protein Assay Reagent (Pierce, Rockford, IL), with BSA as a standard. AMPK activity was determined as described (21), except that a 50 mM PIPES buffer was used (18). AMPK activity is expressed as picomoles of phosphate incorporated into SAMS peptide per milligram of protein in muscle sample supernatant during 1 min.

Statistics. Groups were compared by one-way ANOVA, and statistically significant differences were localized by Bonferroni’s t-test. P < 0.05 was considered significantly different in two-tailed tests.

RESULTS

Experiments with identical total force development. Overall, in experiments on soleus muscle in which total force development was kept constant, 2-DG uptake increased (P < 0.05) with stimulation frequency (Fig. 1). However, 2-DG uptake was identical (P > 0.05) at the two lower (0.25 and 0.5 Hz) as well as at the two higher (1 and 2 Hz) frequencies (Fig. 1). Overall, in these experiments, muscle glycogen concentrations decreased (P < 0.05; Fig. 2), whereas lactate concentrations and AMPK activity increased (P < 0.05; Fig. 2). Again, however, both glycogen concentrations and AMPK activity were identical (P > 0.05) at the two lower (0.25 and 0.5 Hz) as well as at the two higher (1 and 2 Hz) frequencies (Fig. 2); also, lactate concentra-
tions did not differ between 1- and 2-Hz stimulation (P < 0.05; Fig. 2). Concentrations of PCr and ATP decreased (P < 0.05), whereas those of AMP, ADP, and IMP increased (P < 0.05) in response to electrical stimulation (Table 1), the changes in most cases being significantly less marked at 0.25-Hz than at 2-Hz stimulation (Table 1).

Experiments with identical overall metabolic rate. In these experiments, which were carried out on soleus, FDB, and epitrochlearis muscles, resting length and, in turn, total force development were reduced at 2-Hz compared with 0.5-Hz stimulation, resulting in identical amounts of glycogen depletion and lactate accumulation at the two conditions (Figs. 3, 4, and 5). In contrast to the constant force development findings, 2-DG uptake did not differ in soleus, FDB, and epitrochlearis muscles between stimulation at the high and the low frequency (P > 0.05; Figs. 3, 4, and 5). AMPK activity was lower (P < 0.05) at 2 Hz than at 0.5 Hz in soleus muscle (Fig. 3), but it was identical (P > 0.05) at the two stimulation frequencies in both FDB and epitrochlearis muscles (Figs. 4 and 5).

DISCUSSION

Previous studies of both frog (7) and rat (10) muscle have concluded that frequency of stimulation is a major determinant of glucose transport in contracting muscle. The major new finding of the present study is that an increase in stimulation frequency enhances glucose transport only when accompanied by energy depletion and increase in AMPK activity.

In the earlier study of frog sartorius muscle, it was found that only stimulation frequency, and not workload, influences glucose transport during contractions (7). In contrast, we have recently shown that, in rat soleus muscle, at a given stimulation frequency, glucose transport varies directly with total force development.
and accompanying catabolism (9). In the previous study of rat hindlimb muscles in which glucose transport increased with stimulation frequency, mechanical performance was not reported (10). However, contractions were isometric, and total force development probably varied directly with stimulation frequency, rendering difficult an evaluation of the influence of stimulation frequency per se on glucose transport. Therefore, in the present experiments on soleus muscle stimulation, frequency was varied while total force development was kept constant by adjusting resting muscle length. We found that the enhancing effect of stimulation frequency on glucose transport does not depend on force development (Fig. 1).

On the face of it, this finding is in line with the view that glucose transport is stimulated by signals, e.g., Ca^{2+}, arising during the excitation-contraction coupling. Interestingly, however, despite identical total force development, changes in muscle concentrations of glycogen and lactate (Fig. 2) and nucleotides (Table 1), as well as changes in muscle AMPK activity (Fig. 2), were more marked at the two higher than at the two lower of the four applied stimulation frequencies. This decline in mechanical efficiency at the higher stimulation frequencies corresponds with the observation that fatigue was more pronounced at the higher than at the lower frequencies (2). Accordingly, the influence of stimulation frequency on glucose transport might reflect an effect of signals coupled to metabolic demands.

Also in line with this view, glucose transport did not differ between the two lower and the two higher stimulation frequencies (Fig. 1), and metabolic changes, too, were similar between these frequencies (Fig. 2). Furthermore, in separate experiments carried out in soleus, FDB, and epitrochlearis muscles, muscle length and, in turn, total force development were adjusted to secure that even a marked increase in stimulation frequency was not accompanied by further glycolysis or increase in lactate concentration and AMPK activity (Figs. 3, 4, and 5). Again, in the absence of further catabolism, the increase in stimulation frequency did not cause an increase in glucose transport (Figs. 3, 4, and 5). These findings strongly indicate that, in contracting rat muscle, stimulation frequency

### Table 1. High-energy phosphate compound concentrations in contracting muscle

<table>
<thead>
<tr>
<th>Compound</th>
<th>Basal</th>
<th>0.25 Hz</th>
<th>2 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCr</td>
<td>5.5 ± 0.2</td>
<td>2.9 ± 0.2*</td>
<td>2.1 ± 0.2†</td>
</tr>
<tr>
<td>ATP</td>
<td>3.3 ± 0.1</td>
<td>2.3 ± 0.3*</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>ADP</td>
<td>0.49 ± 0.02</td>
<td>0.53 ± 0.03*</td>
<td>0.70 ± 0.03†</td>
</tr>
<tr>
<td>AMP</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.01*</td>
<td>0.15 ± 0.01†</td>
</tr>
<tr>
<td>IMP</td>
<td>0.02 ± 0.01</td>
<td>0.33 ± 0.01*</td>
<td>0.65 ± 0.12†</td>
</tr>
</tbody>
</table>

Concentrations of phosphocreatine (PCr), ATP, ADP, AMP, and IMP are expressed in mmol/kg. Incubated soleus muscles were electrically stimulated to perform repeated tetanic contractions at 0.25 Hz or 2 Hz for a period of 10 min, while resting length was adjusted to achieve identical total force development. Values are means ± SE; n = 5. *Significantly different from basal value (P < 0.05); †significantly different from 0.25-Hz value (P < 0.05).
Fig. 4. Influence of stimulation frequency on 2-DG uptake (A), lactate (B), glycogen (C), and AMPK activity (D) in incubated rat flexor digitorum brevis (FDB) muscle at a constant metabolic rate. Muscles were electrically stimulated at 2 frequencies (0.5 and 2 Hz) for a period of 10 min, while resting length was adjusted to achieve identical glycogen depletion and muscle lactate accumulation. Force was produced during the 0.5-Hz but not the 2-Hz stimulation. Glycogen concentrations are expressed in mmol of glycosyl units. Values are means ± SE; n = 5–6. *Significantly different from basal value (P < 0.05).

Fig. 5. Influence of stimulation frequency on 2-DG uptake (A), lactate (B), glycogen (C), and AMPK activity (D) in incubated rat epitrochlearis muscle at a constant metabolic rate. Muscles were electrically stimulated at 2 frequencies (0.5 and 2 Hz) for a period of 10 min, while resting length was adjusted to achieve identical glycogen depletion and muscle lactate accumulation. Force was produced during the 0.5-Hz but not the 2-Hz stimulation. Glycogen concentrations are expressed in mmol of glycosyl units. Values are means ± SE; n = 5–6. *Significantly different from basal value (P < 0.05).
influences glucose transport via metabolic changes. The studied muscles differed markedly in fiber type composition, indicating that the conclusion applies to all fiber types.

Contractions, as well as hypoxia (5), stimulate glucose transport in muscle, and the two conditions elicit similar changes in intracellular energy status, i.e., reduction in ATP-to-AMP and PCr-to-Cr ratios, and pH (Table 1, Fig. 2). These changes enhance the activity of AMPK, which has been proposed to be a key mediator of non-insulin-stimulated glucose transport (3, 12, 18, 22). The view that AMPK activity is an essential determinant of contraction-induced glucose transport is in line with our previous finding that glucose transport and AMPK activity vary in parallel when force development is varied during muscle stimulation at a given frequency (9). The present study has shown that glucose transport and AMPK activity are also closely correlated when stimulation frequency is varied while force development is kept constant (Fig. 1). This adds to the belief that AMPK is a key regulator of contraction-stimulated glucose transport in general, and furthermore, it also indicates that the specific effect of stimulation frequency is mediated via AMPK.

However, the present study also suggests that AMPK activity is not the only determinant of contraction-stimulated glucose transport. Thus, in experiments on soleus muscles in which stimulation frequency was increased while force development was reduced, glucose transport remained constant in the face of a decrease in AMPK activity (Fig. 3). Muscle glycogen depletion has been proposed to enhance glucose transport (6) but did not differ in these experiments (Fig. 3). Hypothetically, Ca$^{2+}$-mediated mechanisms to some extent substituted for AMPK activity at the higher stimulation frequency. Compatible with this possibility, it has been shown that contraction-stimulated glucose transport can be reduced by various calmodulin and PKC inhibitors (8).

In conclusion, the present study has shown that glucose transport in contracting rat muscle increases with stimulation frequency, but only when this is accompanied by energy depletion and increase in AMPK activity. This indicates that contraction-stimulated glucose transport is more closely related to metabolic demands than to signals arising during the excitation-contraction coupling.

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