

Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation

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Submitted 25 August 2003; accepted in final form 2 October 2003

Kim, Junghoon, Raquel S. Solis, Edward B. Arias, and Gregory D. Cartee. Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation. *J Appl Physiol* 96: 575–583, 2004. First published October 10, 2003; 10.1152/jappphysiol.00909.2003.—Exercise enhances insulin-stimulated glucose transport (GT) in skeletal muscle. Evidence suggests that 5' AMP-activated protein kinase (AMPK) and glycogen may be important for enhanced insulin sensitivity. Our goals were to investigate the effect of various in situ muscle contraction protocols on insulin-stimulated GT and assess the relationship of contraction-induced changes in AMPK and glycogen with postcontraction improvement in insulin-stimulated GT. Rats were anesthetized, both ulnar nerves were exposed, and one nerve was electrically stimulated to contract forelimb muscles. We performed a series of five experiments, sequentially varying only one contraction parameter (train duration, train rate, pulse frequency, number of 5-min bouts, or pulse duration) while holding the others constant. Both epitrochlearis muscles were dissected out and incubated for 3.5 h before measurement of GT. For each contraction parameter studied, we identified an apparent threshold value that did not induce a significant increase in insulin-stimulated GT and an apparent peak value, above which there was a plateau or decline in insulin-stimulated GT. Using other rats, we evaluated muscle AMPK phosphorylation and glycogen concentration immediately postcontraction. AMPK phosphorylation and reduction in glycogen were increased compared with resting controls in each protocol, which had previously been shown to increase insulin-stimulated GT, as well as in several protocols that did not significantly increase insulin-stimulated GT. These data suggest that contraction-induced AMPK phosphorylation and decrease in glycogen may be necessary but are not sufficient for the postcontraction increase in insulin-stimulated GT in rat skeletal muscle.

adenosine 5'-monophosphate-activated protein kinase; exercise; glucose transport

PRIOR EXERCISE CAN INCREASE glucose transport in skeletal muscle via two separate mechanisms. It enhances glucose transport in rat skeletal muscle in the absence of insulin (insulin-independent glucose transport), and it enhances insulin-dependent glucose transport (1, 31). The insulin-independent effect is evident during (22) and immediately after exercise (32). After exercise, the enhanced insulin-independent glucose transport progressively reverses, with little or no residual effect found at ~3 h postexercise in rats (7, 32). At this time, glucose transport activity by a physiological insulin concentration is significantly improved (2, 7, 19, 27, 28, 32). Similar effects on glucose

transport are found after electrically stimulated in situ muscle contraction (7).

Although most studies use only a single exercise or contraction protocol, several researchers have studied effects of varying muscle contraction protocols on insulin-independent glucose transport (9, 11, 13, 16, 20, 22). A consistent finding has been that insulin-independent glucose transport is progressively increased with increasing twitch (20, 22) or train (11, 13) rates. However, at relatively high contraction frequencies, glucose transport plateaus, or even declines, despite further increments in contraction rate.

In contrast to this consensus regarding insulin-independent glucose transport, little is known about the relationship between exercise/contraction protocol and postexercise insulin sensitivity. Apparently, no published studies have systematically characterized the effects of varying electrically stimulated contraction protocols on insulin-stimulated glucose transport.

Previous studies have used different contraction protocols to evaluate the relationship between the increase in insulin-independent glucose transport and processes that are putative regulators of glucose transport (11, 15). Such an approach might also be useful to identify which contraction-related processes are necessary to trigger the subsequent improvement in insulin sensitivity. Stimulation of 5' AMP-activated protein kinase (AMPK) and decreased glycogen levels are consequences of muscle contraction that have been suggested as potentially important factors for enhanced insulin action after exercise. Fisher et al. (6) provided evidence that activation of AMPK can lead to subsequent enhancement in insulin-stimulated glucose transport. Nolte et al. (23) reported that reduction in muscle glycogen, induced by injecting rats with epinephrine, led to a subsequent enhancement of insulin sensitivity measured in isolated muscle.

The first aim of this study was to characterize the effect of various muscle contraction protocols on insulin-stimulated glucose transport. Rather than manipulating only a single contraction parameter, we performed a series of experiments in which we systematically varied pulse duration, pulse frequency, train duration, train frequency, and total minutes of stimulation. Our reasoning was that by manipulating multiple contraction parameters, we would be more likely to reveal a robust relationship between glucose transport and possible regulatory events. Using the results of these experiments, we next examined the relationship between the efficacy of in situ muscle contraction protocols for increasing insulin-stimulated glucose transport and AMPK phosphorylation and glycogen

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concentration. We hypothesized that AMPK phosphorylation and reduction in glycogen concentration would be consistently increased by contraction protocols, which led to improved insulin sensitivity, whereas these changes would not be induced by protocols that did not improve insulin action. Our results indicated that AMPK phosphorylation and decreased glycogen concentration may be necessary, but are not sufficient, for enhanced insulin-stimulated glucose transport after contractile activity.

METHODS

Materials. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). 3-O-[³H]methylglucose and [¹⁴C]mannitol were purchased from NEN (Boston, MA) and Amersham Pharmacia Biotechnology (Piscataway, NJ), respectively. Reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Protein concentrations were measured by using the bicinchoninic acid (BCA; Pierce Biotechnology, Rockford, IL) method. Anti-phospho-AMPK- α (Thr172; catalog no. 2531) was purchased from Cell Signaling (Beverly, MA). Secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) was obtained from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence kits were obtained from Amersham Pharmacia Biotechnology (Piscataway, NJ). Myokinase was from Merck (Darmstadt, Germany). Other reagents were from Sigma Chemical (St. Louis, MO).

Animals. Male specific pathogen-free Wistar rats (Harlan, Indianapolis, IN) were singly housed in wire-bottom cages and provided chow diet (Harlan Teklad Laboratory Rodent Diet, Madison, WI) and water ad libitum. At 1700 on the day before the experiment, food was removed, and the rats were fasted overnight.

Stimulation of muscle to contract in situ. Between 0900 and 1000 on the following day, the animals (body weight of 160–180 g) were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). While under deep anesthesia, animals were placed on a heating pad, both forelimbs were skinned, and the elbow was fixed at an $\sim 90^\circ$ angle. Both ulnar nerves were isolated and connected to subminiature electrodes (Harvard Apparatus, Holliston, MA), and the nerves were transected proximal to the electrodes. A Grass S48 stimulator (Harvard Instruments, Quincy, MA) was used to deliver square-wave pulses (8 V) to one muscle, which was indirectly stimulated to contract via the nerve, and the contralateral muscle, which was treated identically except without electrically stimulated contractions, served a resting control.

Gao et al. (7) demonstrated enhanced insulin sensitivity in epitrochlearis muscles 3.5 h after in situ contraction: 100 pulses/s, 250-ms train duration, 1 train/s train rate, 2 \times 5 min bouts with 1 min of rest, and 0.1-ms pulse duration. We performed a series of experiments in which we sequentially studied the effect of varying only one contraction parameter (e.g., in *experiment 1*, contraction duration was varied) while holding the other contraction parameters constant (train rate, pulse frequency, number of 5-min bouts, and pulse duration). Thus we performed five experiments, each of which characterized the effects of varying only one contraction parameter (Table 1). For each subse-

quent experiment, we began using an effective protocol from the previous experiment (based on the increase in glucose transport in the contracting muscle compared with the paired resting muscle), varying another parameter (e.g., train rate for *experiment 2*) while holding all other parameters constant.

The paired muscles (1 contracting, and the other resting) from each rat were treated identically after dissection. For muscles used to characterize the effect of various stimulation protocols on insulin-stimulated glucose transport, epitrochlearis muscles were dissected out and then incubated as described in *In vitro incubation*. To evaluate AMPK phosphorylation, glycogen, or high-energy phosphates, the muscles were dissected out immediately after contractile activity, freeze clamped by using aluminum tongs cooled to the temperature of liquid N₂, and stored at -80°C until processed as described below.

In vitro incubation. Muscles were dissected out and allowed to recover for 5 min in a flask containing 3 ml of Krebs-Henseleit buffer (KHB) (18) at 35°C . The flasks were placed in a shaking water bath and continuously gassed by using 95% O₂-5% CO₂ during all incubation steps. Muscles were then transferred to another flask containing 3 ml of KHB supplemented with 32 mM mannitol, 8 mM glucose, and 0.1% bovine serum albumin (BSA) and incubated for 3 h at 35°C to allow the insulin-independent effect of contractions on glucose transport to wear off (7, 32). The muscles were next transferred to another flask containing 3 ml of KHB supplemented with 2 mM sodium pyruvate, 36 mM mannitol, and 0.1% BSA with or without 60 $\mu\text{U/ml}$ insulin at 30°C for 30 min.

Measurement of 3-O-[³H]methylglucose transport. After the 30-min incubation with or without insulin, the muscles were transferred to another flask containing 2 ml KHB supplemented with 8 mM 3-O-[³H]methylglucose (0.25 mCi/mmol), 32 mM [¹⁴C]mannitol (6.25 mCi/mmol), and 0.1% BSA with the same insulin concentration as in the preceding step. Muscles were incubated with shaking at 30°C for 10 min. The muscles were next rapidly blotted on ice-cold filter paper, trimmed, freeze clamped with aluminum tongs prechilled to the temperature of liquid N₂, and stored at -80°C until processed. Muscles were weighed and homogenized in 0.3 M perchloric acid, and glucose transport activity was determined as described previously (34). Values are expressed as micromoles per gram per 10 min.

Immunoblotting for phosphorylation of AMPK. Frozen epitrochlearis muscles were weighed, transferred to prechilled glass tubes, and homogenized in ice-cold lysis buffer (1.2 ml/100 mg muscle weight) containing (in mM) 20 mM Tris (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β -glycerophosphate, 1 Na₃VO₄, 1% Triton X-100, and 1 $\mu\text{g/ml}$ leupeptin. Homogenates were rotated for ~ 2 h at 4°C and then centrifuged at 12,000 g for 10 min. Protein concentrations of the supernatants were determined by the BCA method (Pierce, Rockford, IL). Proteins (40 μg) were resolved on a 10% SDS-PAGE gel and transfer buffer overnight at a constant current of 150 mA (20 mM Tris, 150 mM glycine, 0.025% SDS, and 20% methanol). Nitrocellulose blots were incubated in blocking solution (transfer buffer, 0.1% Tween 2 with 5% nonfat dry milk) for 1 h at room temperature and were then washed three times and incubated overnight at 4°C with a primary antibody that detects phosphorylation of AMPK (both α_1 and α_2) at Thr172. Blots were

Table 1. Parameters for each contraction protocol

Experiment No.	Train Duration, ms	Train Rate, train/s	Pulse Frequency, pulse/s	No. of 5-min Bouts	Pulse Duration, ms
1	(25–250)	1	100	2	0.1
2	100	(0.25–2.0)	100	2	0.1
3	100	2	(25–100)	2	0.1
4	100	2	50	(1–4)	0.1
5	100	2	50	2	(0.03–0.3)

Numbers in parentheses represent the range of values tested with all others held constant, as indicated for each experiment.

then washed and incubated with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) for 1 h at room temperature. Blots were then washed of excess antibody and subjected to enhanced chemiluminescence, and immunoreactive protein was quantified by densitometer (Bio-Rad).

Glycogen and high-energy phosphate measurement. Perchloric acid extracts of muscle were prepared as previously described (19). Aliquots were assayed for glycogen by amyloglucosidase method (25) and high-energy phosphates (ATP, PCr, and AMP) as described by Lowry and Passonneau (19).

Statistical analysis. Data are expressed as means \pm SE. A paired *t*-test was used to compare values for contracting muscles with paired, resting muscles. For each experiment, the Pearson product moment correlation was used to evaluate the relationship between contraction-induced changes in insulin-stimulated glucose transport and varying values for contraction parameters (e.g., train duration, etc.). The correlations between contraction-induced changes in insulin-stimulated glucose transport and contraction-induced changes in AMPK phosphorylation and glycogen depletion were assessed by using Pearson's product moment correlation. Individual data that deviated from group mean values by >2 standard deviations were regarded as an outlier and excluded from further statistical analysis (no more than 1–2 samples were excluded from any group). A *P* value of ≤ 0.05 was considered statistically significant. Data were analyzed with Sigma-Stat (SPSS, Chicago, IL).

RESULTS

Experiment 1. For the first experiment, only train duration was varied (between 25 and 250 ms) while other contraction parameters were held constant (Table 1). Data are presented for the contraction-induced increase in glucose transport (value of contracting muscle minus paired resting control value) for insulin-stimulated muscles (Fig. 1A). The mean \pm SE for all resting controls that were insulin-stimulated was $0.340 \pm 0.013 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$ ($n = 225$). The increase in glucose transport for insulin-stimulated muscles postcontraction was significantly greater than paired resting muscles for the following train durations: 50 ms ($0.065 \pm 0.018 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 16$, $P < 0.01$), 100 ms ($0.086 \pm 0.013 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 25$, $P < 0.01$) and 250 ms ($0.085 \pm 0.034 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 10$, $P < 0.01$). The apparent threshold of train duration, which must be exceeded for a significant increase in glucose transport, was ~ 25 ms, and 50 ms clearly exceeded the threshold. Furthermore, the contraction effect on glucose transport appeared to plateau at train durations between 50 and 250 ms.

To confirm that the insulin-independent effect of contraction per se had worn off at 3.5 h postcontraction as previously reported after *in vivo* exercise and *in vitro* muscle contraction (7, 32), other animals underwent the same contraction protocols as in *experiment 1*. Paired muscles ($n = 6$ –17 per protocol) were dissected out and treated identically to the insulin-stimulated muscles except that they were not exposed to insulin *in vitro*. The mean \pm SE for resting controls that were not insulin-stimulated for all experiments was $0.196 \pm 0.010 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$ ($n = 166$). None of the contraction protocols from *experiment 1* caused a significant increase in insulin-independent glucose transport at 3.5 h postcontraction (data not shown).

After measuring the effects of a range of contraction duration protocols on glucose transport, we next evaluated the effects of two of the protocols from each experiment on AMPK phosphorylation and energy metabolites (glycogen, ATP, PCr,

and AMP). From each experiment, we studied one protocol that had not resulted in significantly increased insulin-stimulated glucose transport (i.e., for *experiment 1*, 25 ms) and another protocol that did elicit a significant effect (i.e., for *experiment 1*, 50 ms).

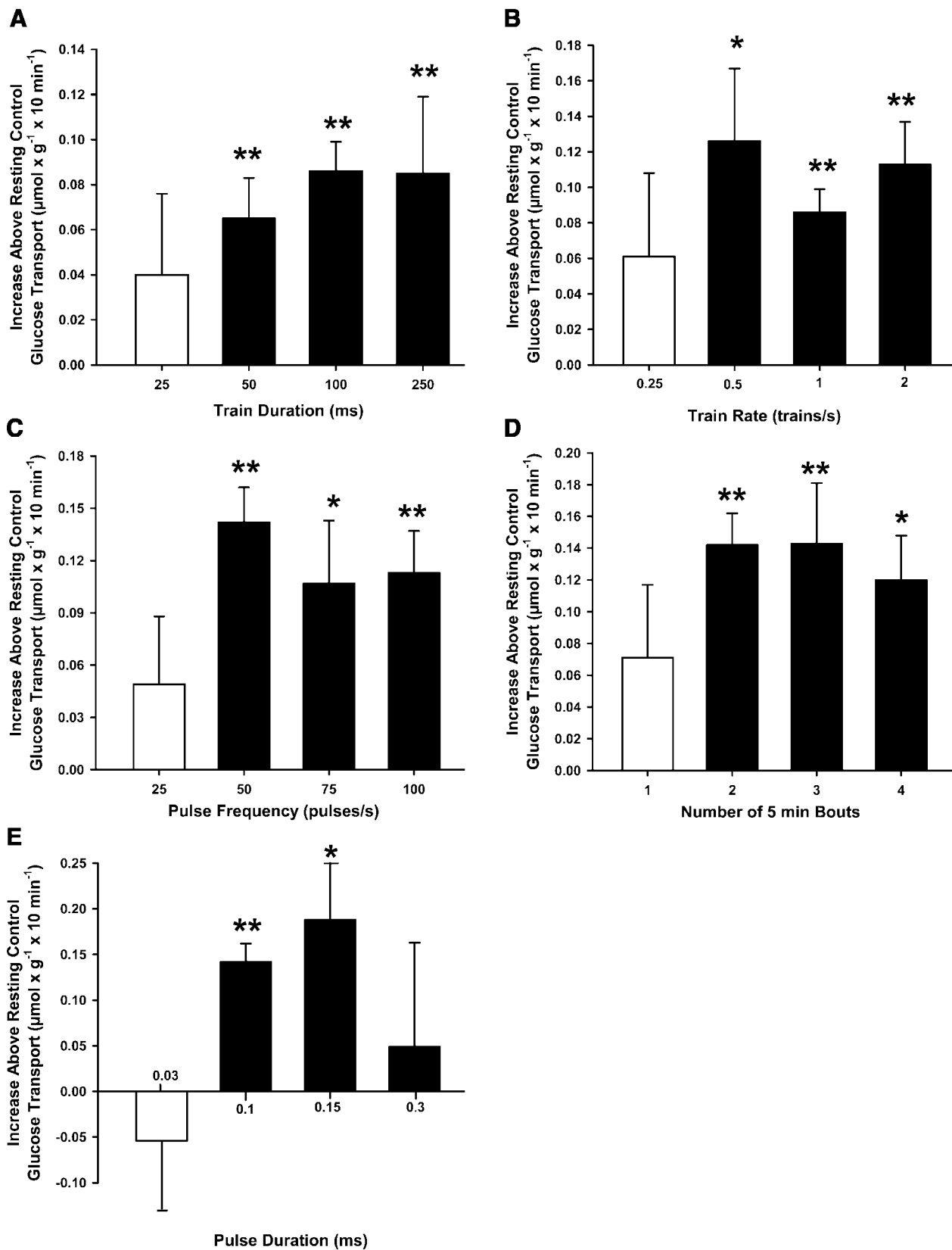
Both the 25- and 50-ms protocols significantly ($P < 0.05$) increased phosphorylation of AMPK compared with paired resting controls (Fig. 2A). Glycogen values (means \pm SE) for all resting control muscles were $14.8 \pm 0.64 \mu\text{mol/g}$ ($n = 38$). Glycogen concentration decreased significantly ($P < 0.05$) with contraction in both protocols studied for *experiment 1* (Table 2). ATP and PCr values for all resting control muscles were 4.53 ± 0.12 ($n = 64$) and $17.15 \pm 0.48 \mu\text{mol/g}$ ($n = 64$), respectively. ATP and PCr concentrations were also significantly ($P < 0.05$) decreased below resting values for the 25- and 50-ms protocols (Table 2). AMP values (mean \pm SE) for all resting control muscles were $0.077 \pm 0.005 \mu\text{mol/g}$ ($n = 54$). AMP values were not significantly increased compared with resting controls for either protocol in *experiment 1*, nor was AMP concentration significantly altered by other contraction protocols studied for *experiments 2–5* (Table 2).

Experiment 2. In the next experiment, only train rate (0.25–3 trains/s) was modified with all other parameters held constant (Table 1). Insulin-stimulated glucose transport was increased compared with noncontracting controls for the following train rates (values expressed as increase above paired resting controls): 0.5 ($0.126 \pm 0.041 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 9$, $P < 0.05$), 1 ($0.086 \pm 0.013 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 25$, $P < 0.01$), and 2 ($0.113 \pm 0.024 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 30$, $P < 0.01$) trains/s (Fig. 1B), whereas the 0.25 trains/s train rate did not significantly increase insulin sensitivity ($0.061 \pm 0.047 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 13$).

To determine whether the effect of contraction on insulin-independent effect was gone, other rats were studied by using the same contraction protocols, but the muscles were subsequently incubated without insulin. Insulin-independent glucose transport was significantly higher ($P < 0.01$) than paired resting controls for the 2 trains/s (postcontraction values minus paired resting values = $0.042 \pm 0.014 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 22$). None of the other protocols significantly increased insulin-independent glucose transport at 3.5 h postcontraction (data not shown).

AMPK phosphorylation was determined immediately after contraction by using the 0.25 train/s protocol (which did not significantly increase insulin-stimulated glucose transport) and the 0.5 train/s protocol (which significantly increased insulin-stimulated glucose transport). AMPK phosphorylation was significantly increased above noncontracting controls for the 0.5 train/s protocol ($P < 0.05$) but not for the 0.25 train/s protocol (Fig. 2B). Glycogen and PCr concentrations were significantly decreased only after the 0.5 train/s protocol, and ATP concentration was significantly reduced only after the 0.25 train/s trial (Table 2).

Experiment 3. In the third experiment, pulse frequency was varied between 25 and 100 pulses/s with other contraction parameters held constant (Table 1). The 25 pulses/s trial failed to significantly enhance insulin sensitivity ($0.049 \pm 0.039 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 7$), while the three higher pulse frequencies (50, 75, and 100 pulses/s) significantly increased the glucose transport of insulin-stimulated muscles compared with noncontracting controls treated with insulin (values are



the increase above resting values): $0.142 \pm 0.022 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 38$, $P < 0.01$; $0.107 \pm 0.036 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 13$, $P < 0.05$; and $0.113 \pm 0.024 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 30$, $P < 0.01$, respectively (Fig. 1C).

We also evaluated the effect of each contraction protocol in *experiment 3* on insulin-independent glucose transport at 3.5 h postcontraction. Insulin-independent glucose transport was significantly higher ($P < 0.05$) than paired resting controls for the 25 and 100 pulses/s protocols (postcontraction values minus paired resting values = $0.062 \pm 0.025 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 12$, $P < 0.05$, and $0.042 \pm 0.014 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 22$, $P < 0.01$, respectively). None of the other protocols significantly increased insulin-independent glucose transport at 3.5 h postcontraction (data not shown).

AMPK phosphorylation was significantly ($P < 0.01$) increased compared with noncontracting controls for both the 25 and 50 pulses/s protocols (Fig. 2C). Both of these contraction protocols significantly decreased muscle concentrations of glycogen, ATP, and PCr compared with resting controls (Table 2).

Experiment 4. For *experiments 1–3*, muscles were stimulated to contract for 5 min, followed by 1 min of rest, followed by an additional 5 min of contraction (i.e., 2×5 min). For *experiment 4*, the number of these 5-min contraction periods was varied, and all other contraction parameters were held constant. Between one and four bouts of 5 min were studied, and a 1-min rest period separated each bout (Table 1). The glucose transport for insulin-stimulated muscles postcontraction was significantly greater than paired resting muscles for muscles (values are the increase above resting values) in the 2×5 ($0.142 \pm 0.020 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 38$, $P < 0.01$), 3×5 ($0.143 \pm 0.038 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 10$, $P < 0.01$), and 4×5 min ($0.120 \pm 0.028 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 10$, $P < 0.01$) protocols (Fig. 1D).

There was no significant effect of in situ contraction on insulin-independent glucose transport (values represent the contraction-induced increase above paired resting values) determined 3.5 h after 2×5 and 4×5 min bouts of contraction (data not shown). A significant increase in insulin-independent glucose transport was evident after the 1×5 ($0.049 \pm 0.019 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 6$, $P < 0.05$) and 3×5 min ($0.068 \pm 0.019 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 10$, $P < 0.01$) protocols.

AMPK phosphorylation was significantly ($P < 0.05$) increased compared with noncontracting controls for the 2×5 min protocol but not for the 1×5 min protocol (Fig. 2D). Both of these contraction protocols resulted in significant decreases in muscle concentrations of glycogen, ATP, and PCr compared with resting controls (Table 2).

Experiment 5. In this experiment, we manipulated only pulse duration (between 0.01 and 0.3 ms) and held other parameters constant (Table 1). Insulin-stimulated glucose transport was significantly greater than paired resting muscles (values represent the contraction-induced increase above paired resting values) for the 0.1-ms ($0.142 \pm 0.020 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 38$, $P < 0.01$) and 0.15-ms pulse duration ($0.188 \pm 0.062 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 7$, $P < 0.05$) but not for the 0.03-ms ($-0.054 \pm 0.092 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 10$) and 0.3-ms ($0.049 \pm 0.114 \mu\text{mol}\cdot\text{g}^{-1}\cdot 10 \text{ min}^{-1}$, $n = 8$) protocols (Fig. 1E).

There was no significant remaining effect of in situ contraction per se (values represent the contraction-induced increase above paired resting values) for any of the protocols in *experiment 5*.

Phosphorylation of AMPK was significantly increased ($P < 0.05$) after the 0.03- and 0.1-ms protocols (Fig. 2E). There was a significant decrease in glycogen after the 0.03- and 0.1-ms protocols (Table 2). ATP and PCr concentrations were significantly reduced after the 0.1-ms pulse duration protocol but not after the 0.03-ms pulse duration protocol (Table 2).

Correlations. There was no significant correlation between the contraction-induced change in insulin-stimulated glucose transport and the contraction-induced change in AMPK phosphorylation ($r = 0.130$, $P = 0.758$) or between the contraction-induced change in insulin-stimulated glucose transport and contraction-induced change in glycogen concentration ($r = 0.098$, $P = 0.817$). Contraction-induced increase in insulin-stimulated glucose transport also did not significantly correlate with any of the contraction parameters (train duration: $r = 0.130$, $P = 0.327$; train rate: $r = 0.226$, $P = 0.082$; pulse frequency: $r = 0.116$, $P = 0.366$; number of 5-min bouts: $r = 0.137$, $P = 0.298$; or pulse duration: $r = 0.054$, $P = 0.672$).

DISCUSSION

In this study, we characterized the effects of a number of different muscle contraction protocols on subsequent insulin-stimulated glucose transport. These results, together with measurements of contraction-induced effects on AMPK phosphorylation and glycogen concentration, provided insights regarding these putative modulators of postcontraction insulin-stimulated glucose transport. The most important new results were 1) postcontraction insulin-stimulated glucose transport was responsive to alterations in each of the five contraction parameters that were studied, 2) each contraction protocol that led to significantly increased insulin-stimulated glucose transport and was studied for AMPK phosphorylation and glycogen concentration also had significantly increased AMPK phos-

Fig. 1. Contraction-induced change in glucose transport for rat epitrochlearis muscles incubated with 60 $\mu\text{U/ml}$ insulin. One muscle from each rat was stimulated to contract in situ, with the contralateral muscle serving as resting control. Muscles were dissected out, and after 3.5 h of in vitro incubation they were incubated with 3-*O*-[^3H]methylglucose. Change in glucose transport was calculated by subtracting values for resting control muscles from values from paired muscles that had been stimulated to contract. *A*: variable train duration with other contraction parameters held constant (1 train/s, 100 pulses/s, 2×5 min bouts, and 0.1-ms pulse duration). *B*: variable train rate with other parameters held constant (100 ms, 100 pulses/s, 2×5 min bouts, and 0.1-ms pulse duration). *C*: variable pulse frequency with other parameters held constant (100 ms, 2 trains/s, 2×5 min bouts, and 0.1-ms pulse duration). *D*: variable number of 5-min contraction periods with other parameters held constant (100 ms, 2 trains/s, 50 pulses/s, and 0.1-ms pulse duration). *E*: variable pulse duration with other parameters held constant (100 ms, 2 trains/s, 50 pulses/s, 2×5 min bouts). Data are the same for 100-ms train duration (*A*), 1 train/s train rate (*B*), 2 trains/s train rate (*B*), 100 pulses/s pulse frequency (*C*), 50 pulses/s pulse frequency (*C*), 2×5 min bouts (*D*), and 0.1-ms pulse duration (*E*). Values are means \pm SE ($n = 5$ –38 paired muscles). Glucose transport rate in muscle stimulated to contract compared with paired, resting control: * $P < 0.05$ and ** $P < 0.01$.

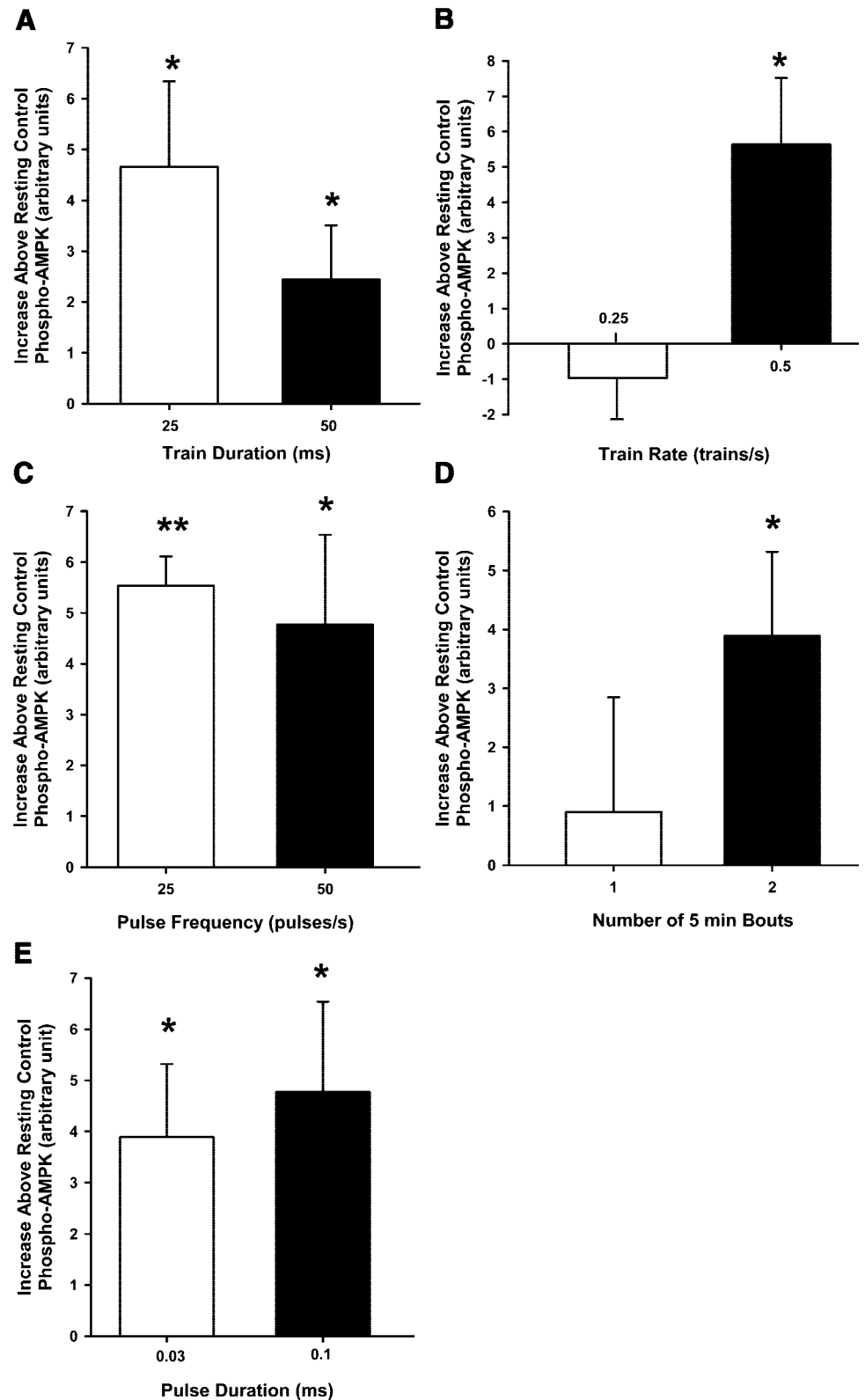


Fig. 2. Contraction-induced change in 5' AMP-activated protein kinase (AMPK) α -subunit phosphorylation (Thr172) for rat epitrochlearis muscles. One muscle from each rat was stimulated to contract in situ, with the contralateral muscle serving as resting control. Muscles were freeze clamped immediately after the contractile activity. Change in AMPK phosphorylation was calculated by subtracting values for resting control muscles from values from paired muscles that had been stimulated to contract. Open bar, protocol did not significantly increase insulin-stimulated glucose transport; shaded bar, protocol significantly increased insulin-stimulated glucose transport (see Fig. 1). A: train duration of 25 and 50 ms. B: train rate of 0.25 and 0.5 train/s. C: pulse frequency of 25 and 50 pulses/s. D: number of 5-min bouts, 1 \times 5 and 2 \times 5 min. E: pulse duration of 0.03 and 0.1 ms. Values are means \pm SE ($n = 6$ –18 paired muscles). Data are the same for 50 pulse/s pulse frequency (C), 2 \times 5 min bout (D), and 0.1-ms pulse duration (E). AMPK phosphorylation in muscles stimulated to contract compared with paired resting control: * $P < 0.05$, ** $P < 0.01$.

phorylation and significantly reduced glycogen concentration, and 3) several contraction protocols that increased both AMPK phosphorylation and decreased glycogen concentration did not significantly enhance insulin sensitivity. These results suggest that some factor(s) other than activation of AMPK phosphor-

ylation and reduced glycogen concentration is important for the contraction-induced improvement in insulin sensitivity.

For each experiment and contraction parameter, we found that the least intense protocol studied did not induce a significant increase in insulin-stimulated glucose transport (Fig. 1).

Table 2. Changes in concentrations of glycogen, ATP, PCr, and AMP in epitrochlearis muscles in different stimulation protocols

Contraction Parameters Varied	Values of Varied Parameters	Increased Insulin Sensitivity	Decrease in Glycogen, $\mu\text{mol/g}$	Decrease in ATP, $\mu\text{mol/g}$	Decrease in PCr, $\mu\text{mol/g}$	Increase in AMP, $\mu\text{mol/g}$
Train duration	25 ms	No	$10.77 \pm 2.00^*$ (4)	$1.22 \pm 0.47^*$ (9)	$4.50 \pm 1.70^*$ (7)	0.014 ± 0.030 (8)
Experiment 1	50 ms	Yes	$7.28 \pm 1.88^*$ (4)	$1.02 \pm 0.41^*$ (11)	$2.92 \pm 1.22^*$ (11)	0.030 ± 0.016 (8)
Train rate	0.25 trains/s	No	3.02 ± 1.98 (6)	$0.74 \pm 0.21^\dagger$ (8)	3.69 ± 1.60 (8)	0.024 ± 0.016 (7)
Experiment 2	0.5 trains/s	Yes	$9.07 \pm 2.28^*$ (4)	0.27 ± 0.21 (8)	$4.37 \pm 0.83^\dagger$ (8)	0.015 ± 0.014 (6)
Pulse frequency	25 pulses/s	No	$13.6 \pm 1.82^\dagger$ (5)	$1.27 \pm 0.44^*$ (9)	$4.21 \pm 1.56^*$ (9)	0.019 ± 0.009 (6)
Experiment 3	50 pulses/s	Yes	$10.34 \pm 2.58^*$ (4)	$2.12 \pm 0.63^*$ (6)	$7.64 \pm 2.65^*$ (6)	0.029 ± 0.020 (6)
No. of 5-min bouts	1	No	$8.68 \pm 1.10^\dagger$ (6)	$1.14 \pm 0.43^*$ (7)	$5.64 \pm 2.16^*$ (7)	0.019 ± 0.021 (7)
Experiment 4	2	Yes	$10.34 \pm 2.58^*$ (4)	$2.12 \pm 0.63^*$ (6)	$7.64 \pm 2.65^*$ (6)	0.029 ± 0.020 (6)
Pulse duration	0.03 ms	No	$8.00 \pm 2.53^*$ (5)	1.37 ± 0.54 (6)	2.57 ± 1.93 (6)	0.021 ± 0.016 (6)
Experiment 5	0.1 ms	Yes	$10.34 \pm 2.58^*$ (4)	$2.12 \pm 0.63^*$ (6)	$7.64 \pm 2.65^*$ (6)	0.029 ± 0.020 (6)

Values are means \pm SE; sample size is provided in parentheses. Values are expressed in $\mu\text{mol/g}$ wet muscle weight. Changes in metabolites were calculated as the difference between contracting muscles and their paired resting controls; thus concentrations of glycogen, ATP, and PCr values represent the contraction-induced decrease, and AMP values represent a trend to increase after contraction. Data are the same for 50 pulses/s pulse frequency in experiment 3, 2×5 min bouts in experiment 4, and 0.1-ms pulse duration in experiment 5. *Significantly different from paired resting controls ($P < 0.05$). † Significantly different from resting controls ($P < 0.01$).

For some of these protocols, there was a trend for contraction to increase the insulin-stimulated glucose transport; however, inspection of the raw data for these groups revealed that a higher value for postcontraction compared with contralateral resting muscles occurred in only 53% of the pairs. In contrast, this percentage was 85% for the protocols with the second-to-lowest intensity in each experiment, and each of these protocols elicited a significant increase in insulin-stimulated glucose transport. It is possible that some of the protocols that did not significantly increase insulin sensitivity were effective in some muscles, but there was clearly not a consistent effect. Accordingly, we have used the term "apparent threshold" for these protocols. We chose to evaluate insulin-stimulated glucose transport at 3.5 h postcontraction based on the results of previous studies (7, 32). However, it is possible that insulin sensitivity can be enhanced at sampling times other than 3.5 h postcontraction by some protocols that failed to induce significant improvements in insulin-stimulated glucose transport when we made the glucose transport measurements.

For each of the dose-response relationships, the contraction-induced increase in insulin-stimulated glucose transport reached a peak value, and further increments in the contraction parameter failed to elicit additional increments in insulin-stimulated glucose transport (Fig. 1). These findings are reminiscent of the results reported by Ruderman and coworkers (8, 27, 35) who published several studies in which rats performed treadmill running at moderate intensity (i.e., 18 m/min for 43 min, then 31–36 m/min for 2 min) or high intensity (i.e., 10 bouts of running at 36 m/min for 5 min/bout with 2–3 min of rest between bouts). Both running protocols led to a significant increase in insulin sensitivity measured postexercise, and the relative magnitude of the improvement appeared to be roughly similar for the two protocols.

It has been hypothesized that improved insulin sensitivity after exercise may be related to a reduction in glycogen concentration (3, 27). One idea is that GLUT-4 glucose transporter proteins are physically associated with glycogen particles and that this association might interfere with GLUT-4 translocation into the cell membrane (3). If this premise is correct, lowering muscle glycogen via exercise would be predicted to improve the availability of free GLUT-4 vesicles,

making the transporter more accessible to the cell membrane (3). Supporting the idea that decreased glycogen concentration is important for insulin sensitivity, Nolte et al. (23) reported that injecting rats with epinephrine induced a decrement in glycogen concentration concomitant with increased insulin-stimulated glucose transport activity. Therefore, we evaluated glycogen concentration in muscles with several contraction protocols that did not elicit a significant increase in insulin-stimulated glucose transport and in protocols that differed in only one contraction parameter but resulted in improved insulin-stimulated glucose transport. Muscle glycogen concentration was significantly reduced by each of the protocols that induced an increase in insulin-stimulated glucose transport and for which glycogen was measured. However, muscle glycogen concentration was also significantly reduced in four of the five protocols that did not cause a significant improvement in insulin-stimulated glucose transport. Several of the protocols of relatively lower stimulation intensity (e.g., 25- compared with 50-ms train duration) resulted in metabolic disturbance (decreased glycogen, ATP, and PCr), which was comparable to the higher intensity stimulation protocol. This is not an unprecedented finding: Johnson and Sieck (14) reported that when rat skeletal muscle was electrically stimulated to contract via the nerve, glycogen, especially in type IIb fibers, was reduced to a greater extent with some lower compared with higher intensity protocols. By studying the effects of nerve and direct muscle stimulation, they demonstrated that the relatively modest decrements in glycogen with higher intensity nerve stimulation were attributable to neuromuscular transmission failure.

It is notable that the magnitude of the decrements in glycogen in several of these protocols was very similar regardless of whether insulin sensitivity was improved. In addition, we found no significant correlation between the decrease in glycogen concentration and the increase in insulin-stimulated glucose transport. These results argue against a decrease in glycogen concentration being the sole cause for enhanced insulin action after exercise, but they do not eliminate the possibility that a decrement in glycogen concentration could modulate other events associated with contraction. For example, activation of AMPK by contraction appears to be influenced by muscle glycogen concentration (4).

In addition to reduction in glycogen, muscle contraction is accompanied by many other cellular events that could influence insulin-stimulated glucose transport. Considerable evidence supports a role for activation of AMPK in the insulin-independent increase in glucose transport after muscle contractions (29, 33). Many studies have shown that glucose transport is increased by incubation of isolated skeletal muscles with 5-aminoimidazole-4-carboximide-1- β -D-ribofuranoside (AICAR), a compound that is taken up by muscle cells and converted to ZMP, an analog of AMP. ZMP activates AMPKK, leading to increased phosphorylation of AMPK, and ZMP also causes the allosteric activation of AMPK. A recent study by Fisher et al. (6) provides evidence that activation of AMPK could also be important for enhanced insulin sensitivity. Isolated rat epitrochlearis muscles were incubated in the presence of serum and AICAR. Fisher et al. (6) reported that, under the conditions of their experiment, AICAR did not induce a change in glycogen concentration, but it did induce a subsequent increase in insulin-stimulated glucose transport. These findings suggest that some factor(s), presumably activation of AMPK, can enhance insulin sensitivity without causing a decrease in muscle glycogen concentration.

Therefore, we determined the effect of several contraction protocols on AMPK phosphorylation on Thr172 of α -subunits. After in situ contraction, phosphorylation of AMPK is significantly correlated with immunoprecipitated AMPK activity of skeletal muscle (24). AMPK phosphorylation was significantly increased for each of the protocols, which resulted in an increase in insulin-stimulated glucose transport. AMPK phosphorylation was also increased in three of the five protocols that did not significantly elevate insulin-stimulated glucose transport. The contraction-induced increase in AMPK phosphorylation was not significantly correlated with the increase in insulin-stimulated glucose transport. As with decreased glycogen concentration, increased AMPK phosphorylation may be involved in elevated postcontraction insulin sensitivity, but it does not appear to be the sole cause. Furthermore, three protocols elicited significant changes in glycogen concentration and AMPK phosphorylation, yet they did not significantly enhance insulin-stimulated glucose transport.

AMPK is a heterotrimeric protein, including one catalytic subunit (α) and two regulatory subunits (β and γ). The antibody we used recognizes phosphor-Thr172 in both α_1 - and α_2 -subunits, so our results are indicative of total AMPK phosphorylation. Therefore, it is possible that contraction protocols with similar levels for total AMPK phosphorylation might differ with regard to activation of specific isoforms. Several studies (5, 12, 30) have indicated that the α_2 -isoform compared with the α_1 -isoform has a lower threshold for activation in response to in situ contraction or in vivo exercise. In situ electrical stimulation (5 trains/s, 100-ms trains, 50 twitches/s, up to 5-min duration) of rat hindlimb muscle via the nerve resulted in increased α_2 -AMPK activity concomitant with no change in α_1 -AMPK activity. Approximately 50% of the increase was evident after only 20 s of contractile activity. Durante et al. (5) reported that 10 min of treadmill running (5 min at 16 m/min, 15% grade, followed by 5 min at 31 m/min, 15% grade) caused a significant increase in α_2 -AMPK of the red quadriceps but not the white quadriceps of rats, concomitant with no change in α_1 -AMPK in either muscle region. Jessen et al. (12) reported that 60 min of treadmill running at

an unspecified speed resulted in significant increases in α_2 -activity in the epitrochlearis, extensor digitorum longus, and soleus concomitant with no change in α_1 -activity and an increase in total AMPK phosphorylation of the epitrochlearis and extensor digitorum longus.

In addition to activation by covalent modification (phosphorylation of α -subunit on Thr172), AMPK can be regulated by allosteric mechanisms, including activation by AMP, and its activity is opposed by high levels of ATP and PCr (17, 33). Therefore, we also measured ATP, PCr, and AMP concentration. Muscle ATP and PCr concentrations were significantly decreased after all but one of the protocols that increased insulin-stimulated glucose transport (i.e., ATP concentration did not decrease in *experiment 2*). Among the five protocols that did not significantly increase insulin-stimulated glucose transport, four resulted in a significant reduction in ATP and three resulted in a significant decline in PCr. For several protocols, PCr levels were only moderately lower in muscles postcontraction compared with resting controls, concomitant with substantial decrements in ATP. The ~1- to 2-min interval between the end of electrical stimulation and freeze clamping of the muscle would be expected to allow significant resynthesis of PCr because of the rapid half-time for PCr resynthesis (21). Therefore, our values represent an underestimate of the contraction-induced PCr decrement. The half-time for resynthesis of ATP is ~15–20 min, and a 1- to 2-min delay would be too brief for significant resynthesis of ATP (21). The brief delay would also not be expected to result in substantial changes in postcontraction alterations in glycogen or AMPK phosphorylation (26).

Although postcontraction total AMP concentration tended to be increased above resting control values, in none of the protocols did this reach statistical significance. Hutber et al. (10) also found that muscle contraction, induced by electrical stimulation of the nerve, did not cause a significant increase in total AMP concentration in gastrocnemius muscle. However, they reported that, despite the lack of a significant increase in total AMP, estimated free AMP concentration was substantially and significantly increased postcontraction. It seems quite likely that free AMP concentration was increased by some or all of the protocols that we used. Regardless, it is notable that one of the protocols that did not cause a significant increase in insulin-stimulated glucose transport (*experiment 1*, 25-ms train duration) resulted in a significant decrease in glycogen, ATP, and PCr along with significant enhancement of AMPK phosphorylation. These results suggest that some other factor(s) associated with muscle contraction is important for elevated insulin sensitivity.

In conclusion, our first aim was to characterize the effect of various muscle contraction protocols on insulin-stimulated glucose transport. As expected, the results allowed us to identify a number of protocols that differed with regard to their efficacy for improving insulin sensitivity. This information allowed us to probe the relationship between increased insulin-stimulated glucose transport and two putative modulators of this effect: glycogen concentration and AMPK phosphorylation. The results supported the first part of our hypothesis, i.e., AMPK phosphorylation and decreased glycogen concentration were consistently increased by contraction protocols that induced an increase in insulin sensitivity. However, the results did not support the second part of our hypothesis, i.e., that

these changes would not occur with protocols that did not increase insulin-stimulated glucose transport. Instead we found three protocols that did not cause a significant improvement in insulin sensitivity but resulted in changes in AMPK phosphorylation and glycogen, which were similar to the changes found after protocols that induced improved insulin action. Taken together, our results indicate that AMPK phosphorylation and reduction in glycogen may be necessary but are not sufficient for enhanced insulin-stimulated glucose transport after contractile activity.

GRANTS

This study was supported by National Institute on Aging Grant RO1 AG-10026 (to G. D. Cartee).

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