Contraction-induced intracellular signals and their relationship to muscle GLUT-4 concentration

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Yaspelkis, B. B., III, A. L. Castle, R. P. Farrar, and J. L. Ivy. Contraction-induced intracellular signals and their relationship to muscle GLUT-4 concentration. Am. J. Physiol. 272 (Endocrinol. Metab. 35): E118–E125, 1997.—This investigation used a model of increased skeletal muscle contractile activity to evaluate whether the adenylate cyclase-adenosine 3',5'-cyclic monophosphate (cAMP) pathway and/or the high-energy phosphate state of the muscle might be temporally related to the contraction-induced increase in skeletal muscle GLUT-4 protein concentration. Plantaris and gastrocnemius muscles of Sprague-Dawley rats were subjected to 3, 7, 14, or 28 days of chronic low-frequency electrical stimulation (10 Hz, 24 h/day). GLUT-4 protein concentration was slightly reduced after 3 days of electrical stimulation, similar to control values at 7 days and significantly elevated above control at 14 days (53%, P < 0.05) and 28 days (338%, P < 0.05) of stimulation. ATP, creatine phosphate, creatine, and Pi were inversely related to GLUT-4 protein concentration. Adenylate cyclase activity increased with electrical stimulation and was significantly related to the increased GLUT-4 protein. cAMP was significantly increased at 14 days of stimulation and remained elevated through 28 days. These results demonstrate that both the adenylate cyclase-cAMP pathway and the high-energy phosphate state of the muscle are temporally related to elevations in skeletal muscle GLUT-4 protein concentration in response to chronic low-frequency electrical stimulation and, as such, suggest that both may comprise a component of the intracellular signal that regulates the contraction-induced increase in skeletal muscle GLUT-4 protein concentration.

chronic low-frequency electrical stimulation; glucose transporter; adenylate cyclase; adenosine 3',5'-cyclic monophosphate; ATP; creatine phosphate; creatine; inorganic phosphate

IT IS WELL ESTABLISHED that insulin accelerates glucose transport by stimulating the translocation of the glucose transporter isoform GLUT-4 from an intracellular pool to the plasma membrane. Exercise training further accelerates the rate of insulin-stimulated skeletal muscle glucose transport and appears to be related to an increased muscle GLUT-4 protein concentration (3, 6, 36, 41). However, like the oxidative enzymes, these exercise-induced improvements are restricted to only those muscles that are recruited during the exercise training (3, 6, 11, 19). It is of interest, therefore, to determine whether a specific contractile effect exists that mediates these improvements in the glucose transport system. An experimental model that increases skeletal muscle contractile activity in the absence of systemic or hormonal effects is chronic low-frequency electrical stimulation. A particularly attractive aspect of this model is that recent reports have shown that chronic low-frequency electrical stimulation increases skeletal muscle GLUT-4 protein content as well as several oxidative enzymes (13, 16, 22). However, the signal that skeletal muscle contractile activity generates to increase muscle GLUT-4 protein concentration is unknown at this time.

Several intracellular signals have been proposed to constitute the “contraction signal.” One proposed signal involves the adenylate cyclase-adenosine 3',5'-cyclic monophosphate (cAMP) pathway (48). In support of this proposed pathway is the observation by Kraus et al. (24) that, during chronic low-frequency stimulation, gene expression of muscle oxidative enzymes correlated temporally and positively with an increase in cAMP and adenylate cyclase activity. However, a similar relationship between the adenylate cyclase-cAMP pathway and increase in GLUT-4 protein during muscle contraction has yet to be established.

An alternative signal by which muscle contraction may initiate increases in skeletal proteins of oxidative metabolism and possibly GLUT-4 has been proposed to involve alterations in the high-energy phosphate state of the skeletal muscle (15, 38). Support for this hypothesis arises from the observation that decreasing the level of high-energy phosphates in skeletal muscle stimulates an increase in the muscle's oxidative capacity and GLUT-4 protein concentration (28, 98, 42). It remains to be determined, however, whether these skeletal muscle adaptations are temporally related to alterations in the high-energy phosphate state during muscle contraction.

Thus the focus of this investigation was to evaluate whether an increase in skeletal muscle contractile activity alters the high-energy phosphate state of the muscle and/or components of the adenylate cyclase-cAMP signaling pathway and whether one or both of these potential signaling pathways are related to elevations in the skeletal muscle GLUT-4 protein concentration. A unique aspect of this investigation was that both of the proposed signaling pathways were evaluated concurrently during various periods of chronic low-frequency electrical stimulation. This differs from previous investigations in which the adenylate cyclase-cAMP signaling pathway (23, 24) and changes in the high-energy phosphate state of the muscle (15, 38) were evaluated independently.

METHODS

Experimental animals. Twenty-nine female Sprague-Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) at ~6–8 wk of age and randomly assigned to one of five groups on the basis of duration of stimulation: 1) control (no stimulation; n = 7), 2) 3 days (n = 5), 3) 7 days (n = 5), 4) 14 days (n = 5), and 5) 28 days (n = 7). All rats were
housed individually and provided laboratory chow and water ad libitum. The temperature of the animal room was main-
tained at 21°C and an artificial 12:12-h light-dark cycle was set. All procedures were approved by the University of Texas at Austin Animal Care and Use Committee and conformed with the guidelines for use of laboratory animals published by the U. S. Department of Health and Human Resources.

Four of the control animals were instrumented for chronic electrical stimulation and served as nonstimulated, sham-
operated controls. It was found after 28 days that no signifi-
cant differences existed between the sham-operated and nonoperated control animals for any of the variables mea-
sured. Therefore, the data were collapsed to form one control group.

Surgical procedures for chronic electrical stimulation. Rats
were anesthetized with an intraperitoneal injection of anes-
thesia cocktail (0.8 ml/kg) that contained ketamine HCl (100
mg/ml), xylazine-HCl (20 mg/ml), and acepromazine (10
mg/ml). Once the rat had been anesthetized, the lateral side
of the right hind limb and the top of the head were trimmed
free of fur. Under aseptic conditions, an incision ~2 cm in
length was made through the skin on the lateral aspect of
the right hind limb ~0.5 cm below and parallel to the femur. The
skin was reflected back and an opening was made between
the two heads of the biceps femoris by blunt dissection. The
two heads of the biceps femoris were reflected back, and the
 Tibial nerve and sutured in place. The two
nerve was gently separated from the surrounding tissue
and exposed. A small bipolar cuff, which was fashioned from
Silastic tubing and fine multistranded medical-grade stain-
less steel wire (ASG92, Cooner Wire, Chatsworth, CA), was
placed around the tibial nerve and sutured in place. The two
heads of the biceps femoris and skin were sutured in layers.
The electrodes wires from the bipolar cuff were led subcuta-
neously to the head, where they exited via a tether-swivel
system that has previously been described by Westgaard and
Lomo (47). The tether-swivel system was equipped with a
potentiometer, which allowed the chronic electrical stimu-
lation to be individually adjusted for each animal on a daily
basis to the maximal level that the animals could tolerate
without signs of discomfort (i.e., normal cage activity and
grooming). The rats were allowed to recover for 5 days after
surgery before the electrical stimulation was implemented.
The rats were stimulated for 24 h/day with a Stoelting
stimulator (Chicago, IL), which was set to produce constant
bipolar square-wave pulses of 250-us duration and a fre-
quency of 10 Hz.

Tissue collection. The rats were anesthetized with an
intraperitoneal injection of pentobarbital sodium (6.5 mg/100
 g body wt). The animals that were undergoing chronic
electrical stimulation were unplugged from the stimulation
system for injection, immediately reattached to the system,
and checked to ensure that the triceps surae group was
contracting. Contraction of the triceps surae group was
allowed to continue for a minimum of 5 min after injection of
the anesthetic. After this standardized period of contraction,
the skin was reflected away from the leg, the soleus was
isolated and removed, and then the plantaris and gastrocne-
mus (P/G) were freeze clamped in situ with tongs cooled in
liquid N2. Muscles from the stimulated animals were
 
coll ected during contraction between hours 10 and 12 of the 24-h
stimulation period. The P/G muscles were freeze clamped
together and collected on days 0, 3, 7, 14, or 28 of the chronic
electrical stimulation period. All muscles were stored at
−80°C until they were analyzed.

Determination of skeletal muscle GLUT-4 protein concentra-
tion. Muscle samples were weighed frozen and then homog-
enized (VirTishear, Gardner, NY) in HES buffer (20 mM
N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1 mM
EDTA, and 250 mM sucrose; pH 7.4: 1:20 wt/vol) using three
15-s bursts at high speed. The protein concentration of the
homogenate was determined by the Bradford method (5).
Samples were diluted 1:1 with Laemmli (27) sample buffer,
and 75 µg of protein were subjected to sodium dodecyl
sulfate-polyacrylamide gel electrophoresis on a 12.5% resolv-
ing gel using the Mini Protean II system (Bio Rad, Richmond,
CA). Resolved proteins were transferred to polyvinyliden-
e difluoride (PVDF) sheets (Bio-Rad) by the method of Towbin
et al. (46) with a Bio-Rad SD semidytransfer unit, utilizing
the buffer system of Kyhse-Anderson (26). PVDF sheets were
blotted with the affinity-purified polyclonal GLUT-4 antibody
R1184A (donation of Dr. Mike Gibbs, Pfizer Central Research,
Groton, CT) as previously described (12) and detected with
the enhanced chemiluminescence method (Amersham Life
Science, Arlington Heights, IL). Labeled bands were quanti-
fied by capturing images of the autoradiographs in a Macin-
tosh IIci computer (Apple Computer, Cupertino, CA). The
autoradiographs were produced by an image scanner (600
Plus Scanner, Mirror Technologies, St. Paul, MN) equipped
with a transparency module. The captured images were
digitized and imported into imaging analysis software (NIH
Image 1.55, Bethesda, MD) and the densities of the labeled
bands were calculated. Each band was corrected for back-
ground activity and expressed as a percentage of a standard
(30 µg of heart homogenate protein) run on each gel.

Measurement of tissue high-energy phosphate compounds
and cAMP concentration. Portions of the muscles were
pulverized in the frozen state under liquid N2 and extracted with
perchloric acid (PCA) according to the procedure described by
Williamson and Corkey (49). Aliquots of the PCA extracts
were stored at −80°C until they were measured for the
high-energy phosphate compounds, ATP (30), creatine phos-
phate (29), creatinine (4), and P1 (Sigma Diagnostics, Kit no.
670-C, St. Louis, MO).

For tissue cAMP determination, 400 µl of the PCA extracts
were lyophilized and resuspended in 150 µl of DPC (Dia-
nostic Products, Los Angeles, CA) assay buffer. Fifty microliters
of the resuspended solution were then assayed for cAMP
content using a commercially available radioimmunoassay
kit (Diagnostic Products).

Measurement of adenylate cyclase activity. Muscles were
thawed over ice and finely minced in 4 ml of homogenization
buffer [50 mM tris(hydroxymethyl)aminomethane (Tris), 5
mM EDTA, and 90 mM NaCl, pH 7.5] at 4°C. The tissue was
then homogenized and filtered through a layer of silk screen
towel to remove large pieces of connective tissue, and the
filtrate was centrifuged at 40,000 g for 30 min at 4°C. The
supernatant was decanted, and the remaining pellet was
resuspended in 4 ml of resuspension buffer (50 mM Tris, 90
mM NaCl, 1.5 mM MgCl2, and 1.0 mM EDTA, pH 7.5). This
suspension was then centrifuged at 40,000 g for 30 min at
4°C. The pellet, which was assumed to represent the crude
cytoplasmic membrane fraction, was resuspended (~10 −10
mg protein/ml) in 1.5 ml of resuspension buffer. The final
suspension was aliquoted and stored at −80°C until analyzed
for adenylate cyclase activity and protein concentration (5).

Adenylate cyclase activity was determined by adding a
100-µl aliquot of the membrane fraction that was diluted in
resuspension buffer to 0.40 mg protein/ml to a reaction
mixture that contained 0.2 mM ATP, 100 µM GTP, 10 mM
Tris-HCl (pH 7.5), 1 mM MgSO4, 1 mM 3-isobutyl-1-
methylxanthine, 0.1% bovine serum albumin, 25 µM cyclic
adrenaline phosphokinase, 20 U/ml adenosine deaminase, and 10
mM phosphocreatine with one of the following stimulatory
compounds: 100 µM forskolin, 5 mM NaF, or 10 µM isoctril-

enol. These compounds were utilized to assess possible differences in activation of adenylate cyclase activity that may have existed among groups by stimulating the enzyme directly with forskolin, through the guanine-nucleotide regulatory G protein (G) with NaF and through the β-receptor with isoproterenol. The assay reaction was performed for a duration of 15 min at 30°C and then stopped by addition of 100 μl of 10% PCA. The samples were then incubated on ice for 30 min, titrated (pH 5.5-6.5) with 30% (wt/wt) potassium bicarbonate, and centrifuged for 15 min at 4°C (2,000 g). Duplicate 50-μl aliquots of the supernatant were removed for cAMP determination using a commercially available radioimmunoassay kit (Diagnostic Products). Adenylate cyclase activity is expressed as picomoles cAMP per milligram protein per minute.

Statistical analysis. The data were analyzed using a one-way analysis of variance to test the effects of chronic low-frequency electrical stimulation over time. Differences between means were determined with Fisher’s least significant differences post hoc test. Linear regression analysis was conducted between the high-energy phosphates and adenylate cyclase activity to skeletal muscle GLUT-4 protein concentration to obtain correlation coefficients. Differences were considered significant if P values < 0.05 were obtained. All values are expressed as means ± SE.

RESULTS

Body weight and muscle weight. It was observed that the body weight of animals subjected to chronic electrical stimulation did not differ between groups (3 days: 256.0 ± 4.3 g, 7 days: 238.0 ± 8.3 g, 14 days: 245.0 ± 11.6 g, and 28 days: 245.7 ± 3.7 g) or from that of the nonstimulated control animals (control: 252.0 ± 2.7 g). The weight of the P/G muscles that were electrically stimulated for 3 days (1.27 ± 0.09 g), 7 days (0.88 ± 0.08 g), 14 days (0.88 ± 0.20 g), and 28 days (0.62 ± 0.09 g) was significantly less than the nonstimulated controls (1.44 ± 0.15 g) but did not differ among the stimulated groups. The reduction in muscle weight found in the present investigation is consistent with previously published reports that have utilized chronic electrical stimulation as a model of increased skeletal muscle contractile activity (13, 35).

Skeletal muscle GLUT-4 protein concentration. GLUT-4 protein concentration (Fig. 1) was significantly elevated after 14 (53%) and 28 days (338%) of stimulation compared with control animals. In addition, the GLUT-4 protein concentration in the 28-day group was 186% greater than that of the 14-day group.

Adenylate cyclase activity and tissue cAMP concentration. After either 3 or 7 days of chronic electrical stimulation, adenylate cyclase activity in the P/G muscles was not significantly different from the control group (Fig. 2). However, after 14 and 28 days of electrical stimulation, adenylate cyclase activity was significantly greater, regardless of the stimulatory agent utilized to assess activity, compared with control, 3-day, and 7-day groups. Basal and forskolin- and NaF-stimulated adenylate cyclase activity was not different between the 14- and 28-day groups, but isoproterenol-stimulated adenylate cyclase was significantly greater at 28 days than at 14 days.
CONTRACTION-INDUCED SIGNALS AND MUSCLE GLUT-4 CONCENTRATION

El21

600

Control 7d 14d 28d

Time

Fig. 3. cAMP concentration in plantaris and gastrocnemius muscles from female Sprague-Dawley rats subjected to various durations of continuous (24 h/day) low-frequency (10 Hz) chronic electrical stimulation via the tibia1 nerve. *Significantly different from control (P < 0.05); t significantly different from 3 days (P < 0.05).

below control values. After 28 days of chronic low-frequency electrical stimulation, all of the high-energy phosphate compounds were significantly lower compared with the control, 3-day, and 7-day stimulation groups. Additionally, muscle ATP and P1 levels were found to be significantly lower than the 14-day group after 28 days of stimulation.

Relationship of skeletal muscle high-energy phosphates, intracellular metabolites, and adenylate cyclase activity to GLUT-4 protein concentration. ATP, creatine phosphate, creatine, and Pi were inversely related to the skeletal muscle GLUT-4 protein concentration during the chronic electrical stimulation (Fig. 4). Adenylate cyclase activity, regardless of how stimulated, was directly related to the GLUT-4 protein concentration (Fig. 5).

DISCUSSION

The aim of this investigation was to use a model of increased skeletal muscle contractile activity that was independent of systemic or hormonal changes to examine two proposed signaling pathways associated with increases in protein synthesis and to investigate whether one or both of these pathways are temporally related to the contraction-induced increase in skeletal muscle GLUT-4 protein concentration. The two proposed signals investigated were the high-energy phosphate state of the muscle and the adenylate cyclase-cAMP pathway.

Chronic electrical stimulation increased skeletal muscle GLUT-4 protein concentration, which is in agreement with previous observations (13, 16, 22). Of interest was the pattern of change for the muscle GLUT-4 protein concentration during the electrical stimulation. GLUT-4 protein concentration declined after 3 days of stimulation, returned to control levels by 7 days, and then was substantially increased at 14 and 28 days of stimulation. Furthermore, these changes in GLUT-4 protein concentration were inversely related to the high-energy phosphate state of the muscle and directly related to the muscle adenylate cyclase activity. The results suggest that both the high-energy phosphate state of the muscle and the adenylate cyclase-cAMP pathway may comprise a component of the intracellular signal that regulates the contraction-induced increase in skeletal muscle GLUT-4 protein concentration.

The pattern of change in GLUT-4 protein in the present study appears to differ from that observed by Kong et al. (22), who reported that 1 day of electrical stimulation elevated skeletal muscle GLUT-4 protein concentration ~2.5-fold. However, Kong et al. reported that skeletal muscle GLUT-4 protein concentration was unchanged between days 1 and 5 of stimulation. It is unknown, therefore, whether this lack of change in GLUT-4 protein concentration was due to the glucose transporter concentration remaining constant during this time or whether a pattern of change similar to that which we observed in the present investigation occurred. In addition, our observation that skeletal muscle GLUT-4 protein concentration declines before being increased in response to chronic electrical stimulation parallels the changes that have been observed for citrate synthase activity (17), an oxidative enzyme that normally responds to exercise training in a very similar manner as the GLUT 4 protein (3, 6).

The reason for the initial decline in GLUT-4 protein is unclear, especially because it has been reported that both GLUT-4 mRNA and protein concentration are

Table 1. ATP, CP, creatine, and P1 content in plantaris and gastrocnemius muscle from female rats subjected to electrical stimulation

<table>
<thead>
<tr>
<th>Days of Stimulation</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>5.64 ± 0.46</td>
<td>6.81 ± 0.23</td>
<td>5.10 ± 0.41†</td>
<td>4.25 ± 0.47†</td>
</tr>
<tr>
<td>CP</td>
<td>20.88 ± 1.52</td>
<td>21.37 ± 1.38</td>
<td>16.89 ± 1.72</td>
<td>12.21 ± 1.59‡</td>
</tr>
<tr>
<td>Creatine</td>
<td>6.00 ± 0.81</td>
<td>8.13 ± 1.12*</td>
<td>5.38 ± 0.76†</td>
<td>3.47 ± 0.49‡</td>
</tr>
<tr>
<td>P1</td>
<td>5.07 ± 0.37</td>
<td>5.15 ± 0.21</td>
<td>4.86 ± 0.20</td>
<td>4.03 ± 0.25*‡‡</td>
</tr>
</tbody>
</table>

Values are means ± SE and are in μmol/g wet wt; n = no. of rats. CP, creatine phosphate. Female Sprague-Dawley rats were subjected to various durations of continuous (24 h/day) low-frequency (10 Hz) chronic electrical stimulation via the tibia1 nerve. *Significantly different from control (P < 0.05); †significantly different from 3 days (P < 0.05); ‡significantly different from 7 days (P < 0.05); ‖significantly different from all preceding time points (control -14 days) (P < 0.05).
increased the day after a prolonged bout of exercise (33, 39). However, a plausible explanation is that chronic electrical stimulation is a much more intensive stimulus than exercise training and, as such, may require some metabolic readjusting or remodeling of the skeletal muscle before GLUT-4 protein can begin to increase. This possibility is supported by the recent reports of Asp et al. (1, 2), who demonstrated that intense bouts of eccentric exercise significantly reduced skeletal muscle GLUT-4 protein concentration for up to

Fig. 4. Relationship of ATP (A), creatine phosphate (CP; B), creatine (C), and Pi (D) to skeletal muscle GLUT-4 protein concentration. ATP, CP, creatine, and Pi are expressed as umol/g wet wt. GLUT-4 is expressed as a percentage of heart standard. All time points tested are represented and are presented in Table 1.

Fig. 5. Relationship of adenylate cyclase activity (pmol cAMP·mg⁻¹·min⁻¹) to skeletal muscle GLUT-4 protein concentration. GLUT-4 is expressed as a percentage of heart standard. Adenylate cyclase activity was determined under basal conditions (A), stimulated directly with forskolin (B), through guanine-nucleotide regulatory G protein (Gₙ) with NaF (C), and through β-receptor with isoproterenol (D). All time points tested are represented and are presented in Fig. 2.
2 days before it recovered to preexercise levels. Thus our observation that skeletal muscle GLUT-4 protein concentration declined before it increased with the use of chronic electrical stimulation does not appear to be specific to our experimental paradigm but rather a natural adaptation that occurs in response to intense skeletal muscle activation.

Our observation that alterations in the high-energy phosphate state of the skeletal muscle may mediate, in part, the cellular adaptations that occur in response to chronic contraction support the earlier findings of Green et al. (15). These investigators subjected rabbit tibialis anterior muscles to continuous low frequency electrical stimulation (10 Hz) for 50 days. They observed that the tibialis anterior began to acquire the metabolic profile of a slow-twitch muscle as evidenced by moderate reductions in total adenine nucleotide content, total creatine, ATP, and creatine phosphate, leading these authors to suggest that alterations in the high-energy phosphate state of the muscle reflect a central control element of cellular metabolism and, as such, could be the signal that triggers the contraction-induced adaptation in skeletal muscle. Our finding that the GLUT-4 protein concentration was inversely related to changes in the high-energy phosphate levels extends these earlier findings. Further support for the hypothesis that the high-energy phosphate state of the skeletal muscle initiates alterations in the physiologic profile of the muscle has been provided by investigators who have manipulated the level of high-energy phosphates in the absence of muscle contractile activity with \(\beta\)-guanidinopropionic acid (\(\beta\)-GPA). \(\beta\)-GPA is a structural analog of creatine that reduces skeletal muscle creatine phosphate and ATP levels (14). Consumption of a diet containing 1–2% \(\beta\)-GPA for 6–10 wk has been shown to increase oxidative capacity (30, 42, 47), GLUT-4 protein concentration, and maximal insulin-stimulated 3-O-methylglucose transport (38) in rat skeletal muscle. Thus these observations taken collectively support the hypothesis that alterations in the high-energy phosphate state of the muscle in response to muscle contraction comprise a component of the contraction signal. However, it remains to be determined how this metabolic signal is transferred to the genomic level and initiates the expression of the GLUT-4 protein.

Although it appears that alterations in the high-energy phosphate compounds may provide the impetus for increased skeletal muscle GLUT-4 protein concentration in response to contractile activity, our results also suggest that the adenylate cyclase cAMP pathway may be involved. Exercise training has been shown to increase adenylate cyclase activity of muscle (7, 37), and recently, Kraus et al. (24) reported that it could also be increased by chronic low-frequency electrical stimulation (10 Hz, 24 h/day). In agreement with Kraus et al., we found that adenylate cyclase activity was significantly increased in rat skeletal muscle after 14 and 28 days of stimulation and, in addition, that there was a significant relationship between the increase in adenylate cyclase activity and muscle GLUT-4 protein concentration.

Whether the second messenger of adenylate cyclase, cAMP, was responsible for the changes in skeletal muscle GLUT-4 protein concentration after electrical stimulation is equivocal. GLUT-4 protein concentration was slightly reduced after 3 days and returned to baseline by 7 days of stimulation, whereas the cAMP concentration was unchanged after 3 days and slightly increased by 7 days of stimulation. Likewise, when GLUT-4 protein was dramatically increasing from 14 to 28 days, the cAMP concentration was declining from its highest level of \(666 \pm 91.2\) to \(564 \pm 100\) pmol/g wet wt. Thus the results could be interpreted to indicate that cAMP is not involved in the regulation of GLUT-4 expression and that the significant positive relationship between adenylate cyclase activity and GLUT-4 protein concentration observed was coincidental and not causal in nature.

Another interpretation of the results, however, is that the changes in cAMP preceded the changes in GLUT-4 protein. Therefore, the decline in GLUT-4 protein observed at 3 days may have been preceded by a decline in cAMP, and the return of GLUT-4 protein to baseline from 3 to 7 days may have been preceded by a return of cAMP to baseline on day 3. In a similar manner, the rapid rise in GLUT-4 from 14 to 28 days occurred when the cAMP concentration was declining but was preceded by the highest observed increase in cAMP at 14 days. The decline in cAMP at 28 days as the adenylate cyclase activity and GLUT-4 protein concentration were increasing was not unexpected. Kraus et al. (23) found that adenylate cyclase activity and proteins of oxidative metabolism increased in rabbit tibialis anterior muscle from days 3 to 21 of electrical stimulation. However, the cAMP concentration reached its peak at 10 days and declined toward baseline between days 10 and 21. Kraus et al. suggested that the decline in cAMP was consistent with an increased expression of phosphodiesterase induced by an elevation in cAMP (44) to limit its intracellular level.

Although an increase in intracellular cAMP has not been demonstrated to directly stimulate protein synthesis in response to skeletal muscle contractile activity, cAMP has been found to be involved in gene expression in eukaryotic cells (9, 10, 31, 40, 42). The ability of cAMP to regulate gene expression appears to occur through a conserved nuclear cAMP-responsive enhancer binding protein (CREB), which stimulates the transcription of cAMP-responsive genes (50). Of interest, a CREB has been isolated and shown to transactivate the GLUT-4 protein promoter isolated from a mouse 3T3-L1 library (20). In addition, a CREB has also been shown to transactivate promoters of several genes involved in the metabolism and storage of fats and carbohydrates (32) and the \(\beta\)-adrenergic receptor (9, 10).

The complexity of gene regulation is, however, highlighted by the fact that Kaestner et al. (21) have reported that cAMP downregulates transcription of the GLUT-4 gene in cultured 3T3-L1 adipocytes. Addition-
ally, fasting, which elevates cAMP, has been shown to decrease GLUT-4 mRNA levels in adipose tissue (18). cAMP-induced regulation of GLUT-4 protein expression in cultured 3T3-L1 adipocytes and adipose tissue, however, may differ from that of skeletal muscle GLUT-4 protein regulation. Specifically, GLUT-4 mRNA in rat (8) and mouse (18) skeletal muscle is not lowered by fasting, but rather its expression is increased (8). Moreover, in cultured cells of L6, which is a representative skeletal muscle cell line, the cAMP analog, 8-(4-chlorophenyl-thio)-cAMP, increases hexokinase II gene transcription (34). This finding is significant in that it has been observed that elevations in skeletal muscle hexokinase activity and GLUT-4 protein concentration are coregulated in response to chronic electrical stimulation (22) and exercise training (38). It is unlikely that elevations in cAMP would increase skeletal muscle hexokinase activity while downregulating GLUT-4 protein concentration. Therefore, the possibility exists that the elevated cAMP levels observed in the present investigation may have comprised a part of the signal that initiated the increase in skeletal muscle GLUT-4 protein concentration in response to the electrically induced muscle contraction. This contention is supported by our recent finding that β-adrenergic blockade or downregulation of the β-adrenergic receptor attenuates the exercise training-induced increase in skeletal muscle GLUT-4 protein expression (25). Nevertheless, even if the role of cAMP in gene expression in the present investigation is somewhat inconclusive, our results clearly demonstrate that large elevations in the muscle cAMP concentration do not prevent skeletal muscle GLUT-4 protein concentration from increasing.

In summary, both the adenylate cyclase-cAMP pathway and the high-energy phosphate state of the muscle were found to be temporally related to the elevations in skeletal muscle GLUT-4 protein concentration in response to chronic low-frequency electrical stimulation of the PG group. The results suggest that these proposed signaling pathways may mediate GLUT-4 expression during muscle contraction. However, more research is required to determine the relative importance of each, as well as to confirm their roles as mediators of GLUT-4 expression.

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