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 P_i 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, 38, 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 maintained 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 significant differences existed between the sham-operated and nonoperated control animals for any of the variables measured. Therefore, the data were collapsed to form one control group.

Surgical procedures for chronic electrical stimulation. Rats were anesthetized with an intraperitoneal injection of anesthesia 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 was gently separated from the surrounding tissue and exposed. A small bipolar cuff, which was fashioned from Silastic tubing and fine multistranded medical-grade stainless steel wire (AS632, 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 electrode wires from the bipolar cuff were led subcutaneously 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 stimulation 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-μs duration and a frequency 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 anesthesia. 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 gastrocnemius (P/G) were freeze clamped in situ with tongs cooled in liquid N_2. Muscles from the stimulated animals were collected 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 concentration. Muscle samples were weighed frozen and then homogenized (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% resolving gel using the Mini-Protean II system (Bio-Rad, Richmond, CA). Resolved proteins were transferred to polyvinylidene difluoride (PVDF) sheets (Bio-Rad) by the method of Towbin et al. (46) with a Bio-Rad SD semidry transfer 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 quantified by capturing images of the autoradiographs in a Macintosh IIsi 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 background 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 N_2 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 phosphate (29), creatine (4), and P_i (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 (Diagnostic 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 mesh 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 MgCl, 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 sarcolemmal membrane fraction, was resuspended (~10–50 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 $MgSO_4$, 1 mM 3-isobutyl-1-methylxanthine, 0.1% bovine serum albumin, 25 U/ml creatine 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 isoproter-

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_s) 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 reagent 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.

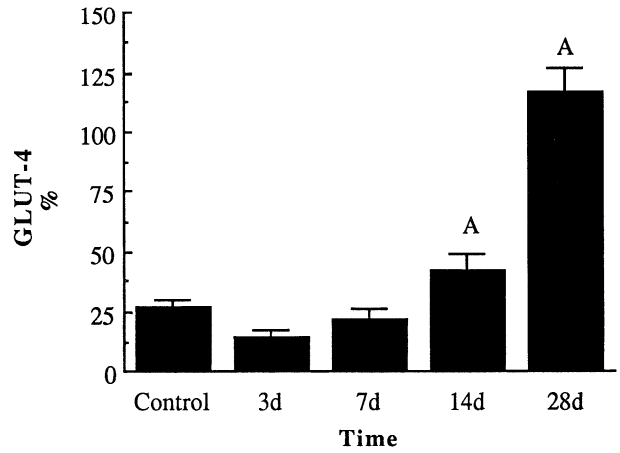

Fig. 1. GLUT-4 protein concentration, expressed as percentage of a heart standard, in plantaris and gastrocnemius muscles from female Sprague-Dawley rats subjected to various durations of continuous (24 h/day) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve. [A]Significantly different ($P < 0.05$) from all preceding time points.

cAMP levels in the P/G muscles were found to be similar to control values over the first 7 days of electrical stimulation (Fig. 3). Although cAMP began to rise slightly (36%) at 7 days of stimulation, it did not become significantly elevated above control values until 14 days of stimulation and then remained significantly different from the control group for the duration of the investigation.

Skeletal muscle high-energy phosphate levels. After 3 days of electrical stimulation, skeletal muscle ATP and creatine levels were found to be significantly elevated above control values (Table 1). After 7 days of electrical stimulation, ATP and creatine had returned to levels similar to control muscle. By 14 days, the high-energy phosphates, in general, exhibited a trend to be reduced

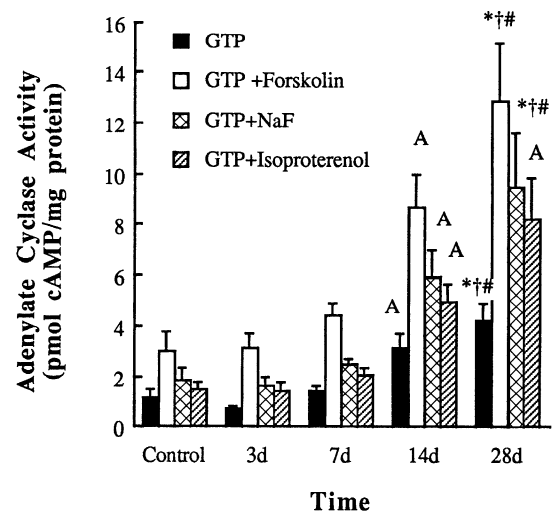

Fig. 2. Adenylate cyclase activity (pmol cAMP·mg^{-1}·min^{-1}) in plantaris and gastrocnemius muscles from female Sprague-Dawley rats subjected to various durations of continuous (24 h/day) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve. *Significantly different from control ($P < 0.05$); †significantly different from 3 days (3d) ($P < 0.05$); #significantly different from 7 days (7d) ($P < 0.05$); [A]significantly different ($P < 0.05$) from all preceding time points.

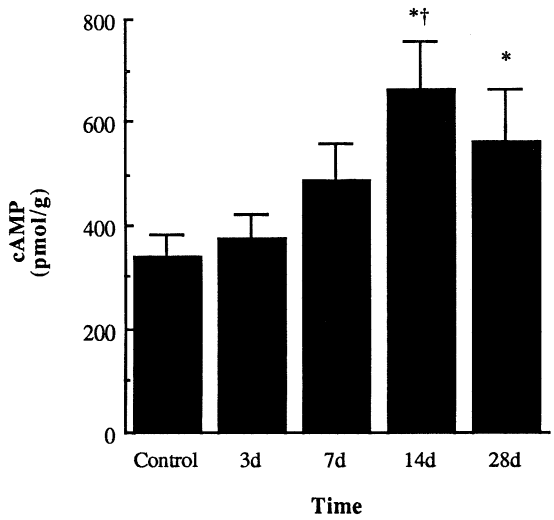

Fig. 3. cAMP concentration in plantaris and gastrocnemius muscles from female Sprague-Dawley rats subjected to various durations of continuous (24 h/day) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve. *Significantly different from control ($P < 0.05$); †significantly different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

		Days of Stimulation			
	Control	3	7	14	28
n	7	5	5	5	7
ATP	5.64 ± 0.46	6.81 ± 0.23	5.10 ± 0.41†	4.25 ± 0.47†	2.04 ± 0.23§
CP	20.88 ± 1.52	21.37 ± 1.38	16.89 ± 1.72	12.21 ± 1.59*†	7.67 ± 1.60*†‡
Creatine	6.00 ± 0.81	8.13 ± 1.12*	5.38 ± 0.76†	3.47 ± 0.49†	2.36 ± 0.48*†‡
P_i	5.07 ± 0.37	5.15 ± 0.21	4.85 ± 0.20	4.03 ± 0.25*†‡	2.97 ± 0.31§

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 tibial 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) ($P < 0.05$).

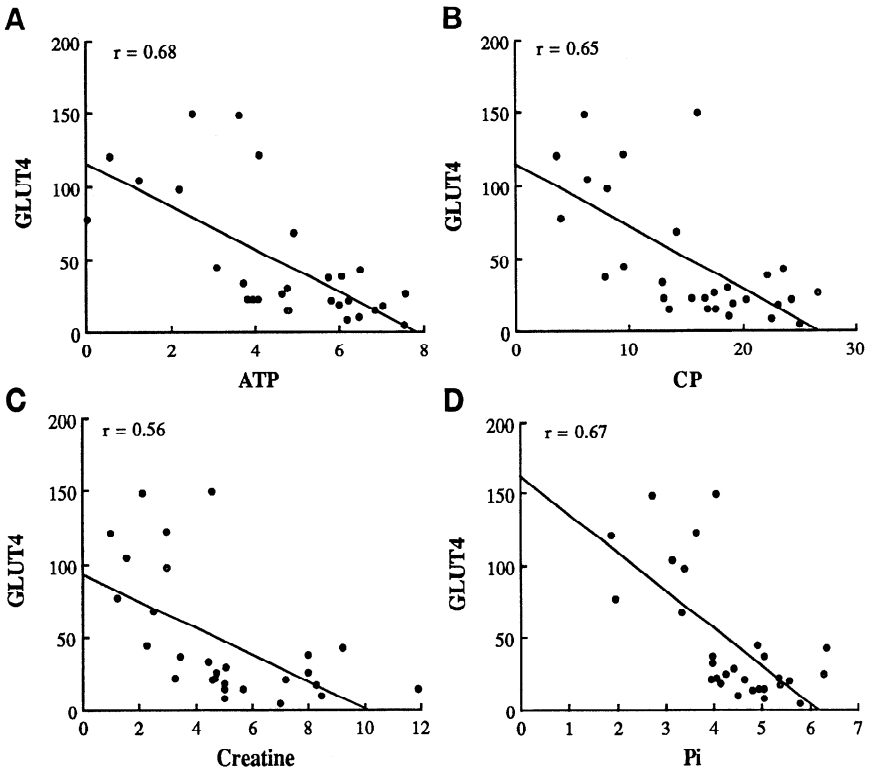

Fig. 4. Relationship of ATP (*A*), creatine phosphate (CP; *B*), creatine (*C*), and P_i (*D*) to skeletal muscle GLUT-4 protein concentration. ATP, CP, creatine, and P_i are expressed as μmol/g wet wt. GLUT-4 is expressed as a percentage of heart standard. All time points tested are represented and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

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

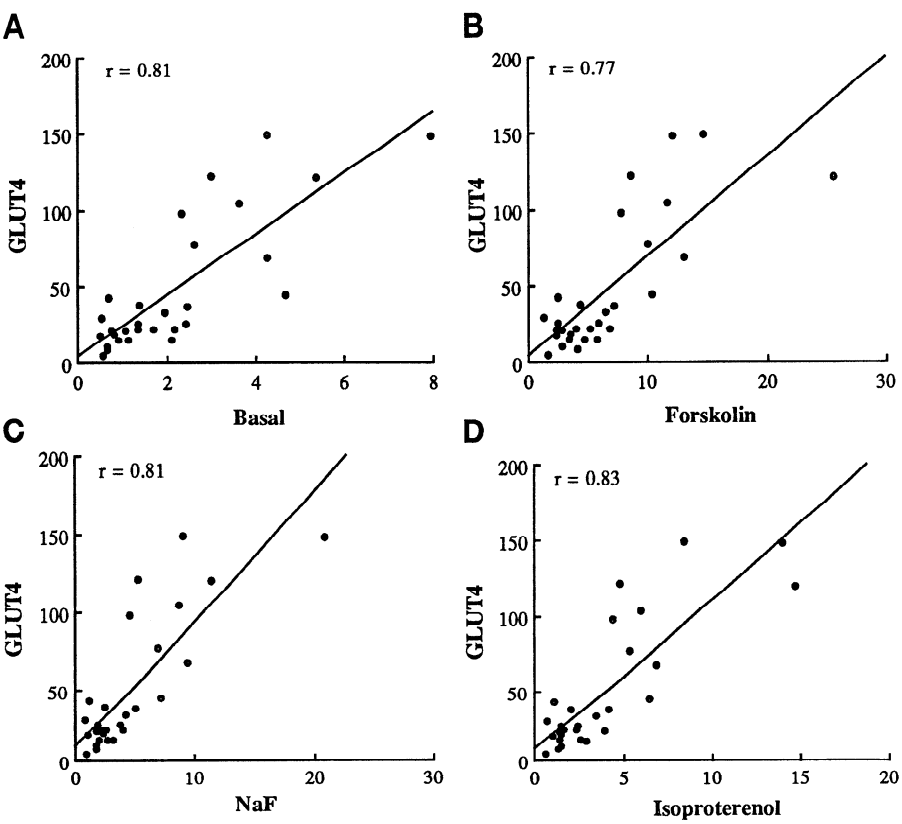

Fig. 5. Relationship of adenylate cyclase activity (pmol $cAMP \cdot mg^{-1} \cdot min^{-1}$) to skeletal muscle GLUT-4 protein concentration. GLUT-4 is expressed as a percentage of heart standard. Adenylate cyclase activity was determined under basal conditions (*A*), stimulated directly with forskolin (*B*), through guanine-nucleotide regulatory G protein (G_s) 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 physiological 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 β-guanidinopropionic acid (β-GPA). β-GPA is a structural analog of creatine that reduces skeletal muscle creatine phosphate and ATP levels (14). Consumption of a diet containing 1–2% β-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 ± 91.2 to 564 ± 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 β_2-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 levels, 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 P/G 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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