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Effect of chronic electrical stimulation and β-GPA diet on GLUT4 protein concentration in rat skeletal muscle

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ABSTRACT

The present study investigated whether alterations in the muscle high energy phosphate state initiates the contraction-induced increase in skeletal muscle GLUT4 protein concentration. Sprague-Dawley rats were provided either a normal or a 2% β-guanidinoproprionic acid (β-GPA) diet for 8 weeks and then the gastrocnemius of one hind limb was subjected to 0, 14 or 28 days of chronic (24 h day^{-1}) low-frequency electrical stimulation (10 Hz). The β-GPA diet, in the absence of electrical stimulation, significantly reduced ATP, creatine phosphate, creatine and inorganic phosphate and elevated GLUT4 protein concentration by 60% without altering adenylate cyclase activity or cAMP concentration. Following 14 days of electrical stimulation, GLUT4 protein concentration was elevated above non-stimulated muscle in both groups but was significantly more elevated in the β-GPA group. Concurrent with this greater rise in GLUT4 protein concentration was a greater decline in the high energy phosphates and a greater rise in cAMP. After 28 days of electrical stimulation, GLUT4 protein concentration and cAMP stabilized and was not different between diet treatments. However, the high energy phosphates were significantly higher in the normal diet rats as opposed to the β-GPA rats. These findings therefore suggest that a reduction in cellular energy supply initiates the contraction-induced increase in muscle GLUT4 protein concentration, but that a rise in cAMP may potentiate this effect.

Keywords adenosine 3′,5′-cyclic monophosphate, adenosine triphosphate, adenylate cyclase, creatine phosphate, creatine, high energy phosphate, inorganic phosphate.

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Exercise training has consistently been found to increase the rate of skeletal muscle insulin-stimulated glucose transport and this increase appears to be related to an increased muscle GLUT4 protein concentration (Ploug *et al.* 1990, Banks *et al.* 1992, Rodnick *et al.* 1992, Brozinick *et al.* 1993, Etgen *et al.* 1993a). However, exercise-induced improvements occur only in muscles that are extensively activated during the exercise training (Ivy *et al.* 1989, Cortez *et al.* 1991, Banks *et al.* 1992, Brozinick *et al.* 1993) suggesting that a signal inherent to the increased muscle contractile activity is responsible for these adaptations. Contraction-induced intracellular signals that have been proposed to function in this capacity include alterations in the high energy phosphate state of the skeletal muscle (Shoubridge *et al.* 1985, Lai & Booth 1990, Green *et al.* 1992, Ren *et al.* 1993) and/or changes in components of the β-adrenergic signalling pathway (i.e. β-adrenergic receptor–adenylate cyclase–cyclic AMP (cAMP)) (Williams *et al.* 1984, Kraus *et al.* 1992). We recently investigated whether one or both of these pathways mediate the contraction-induced increase in skeletal muscle GLUT4 protein concentration by subjecting Sprague-Dawley rats to various durations of chronic low-frequency (10 Hz) electrical stimulation (Yaspelkis *et al.* 1997). It was found that both of the proposed signalling pathways exhibited a temporal relationship to the elevations in skeletal muscle GLUT4 protein concentration, and as such, suggested that both pathways may be part of the intracellular contraction signal. However, it could not be determined whether the increase in skeletal muscle GLUT4 protein concentration was because of the pathways initiating their signals in series or parallel.

In light of our previous investigation being unable to discern the relative importance of the two contraction-induced intracellular signalling pathways, the present investigation was undertaken to evaluate whether alterations in the high energy phosphate state of the

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skeletal muscle may initiate the contraction signal. This question was addressed by providing a 2% β-guanadinoproprionic acid diet (β-GPA) to Sprague-Dawley rats for 8 weeks to lower the level of skeletal muscle high energy phosphate compounds and then subjecting one hind limb to either 14 or 28 days of chronic low-frequency (10 Hz) electrical stimulation. β-GPA is a structural analogue of creatine that competes with creatine for transport into muscle and reduces skeletal muscle creatine phosphate and ATP levels (Fitch *et al.* 1975). Consumption of this diet has been shown to increase the oxidative capacity and GLUT4 protein concentration in rat skeletal muscle (Shoubridge *et al.* 1985, Lai & Booth 1990, Ren *et al.* 1993). However, it is unknown whether a β-GPA diet and skeletal muscle contractile activity initiate elevations in skeletal muscle GLUT4 protein concentration through the same intracellular signalling pathway.

MATERIALS AND METHODS

Experimental animals

Forty-eight female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) at ≈6 weeks of age and were randomly assigned to one of four groups: (1) control ($n = 7$), (2) 2% β-GPA diet ($n = 7$), (3) stimulation ($n = 16$) or (4) stimulation + 2% β-GPA diet ($n = 18$). The stimulation and stimulation +2% β-GPA diet groups were further divided into two subgroups based on duration of stimulation: 14 and 28 days. These periods of stimulation were chosen because previous reports indicated that skeletal muscle GLUT4 protein concentration (Yaspelkis *et al.* 1997) and oxidative capacity (Hood & Pette 1989) do not begin to increase above control levels until ≈10–14 days following the onset of electrical stimulation. All rats were housed individually in a room maintained at 21 °C and an artificial 12 h light–dark cycle. The 2% β-GPA diet and stimulation + 2% β-GPA diet groups received chow containing 2% β-GPA for 8–10 weeks. The control and stimulation groups were provided standard laboratory chow during this time. Following the 8–10-week feeding period, the stimulation and stimulation + 2% β-GPA diet groups were instrumented for chronic electrical stimulation and then remained on their respective diets for the duration of the stimulation period. Chow and water were available *ad libitum* for each group. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and conformed with the guidelines for use of laboratory animals published by the US Department of Health and Human Resources.

Surgical procedures for chronic electrical stimulation

Rats were anaesthetised with an intraperitoneal injection of anaesthesia cocktail (0.8 mL kg^{-1}) that contained ketamine–HCl (100 mg mL^{-1}), xylazine–HCl (20 mg mL^{-1}) and acepromazine (10 mg mL^{-1}). Once the rat had been anaesthetised the lateral side of the right hind limb was trimmed free of fur. Under aseptic conditions an incision that was ≈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 multi-stranded medical grade stainless steel wire (AS632, Cooner Wire Co, Chatsworth, CA, USA), 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 & Lomo (1988). 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, 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^{-1} using a Stoelting stimulator (Chicago, IL, USA) 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 anaesthetised with an intraperitoneal injection of pentobarbital sodium (6.5 mg 100 g^{-1} body wt). To allow administration of the anaesthesia, the rats undergoing chronic electrical stimulation were briefly unplugged from the stimulation system (≈5 s). After the injection, they were immediately reattached to the system and contraction of the triceps surae group allowed to continue. Once the rats were completely anaesthetised, which took between 5 and 7 min, the skin over the right hind limb was reflected back, and the gastrocnemius isolated and freeze clamped *in situ* on days 0, 14 or 28 of the stimulation period. Muscles were collected while contracting from the stimulated rats between 10 and 12 h of the 24-h stimulation period. All muscles were stored at −80 °C until analysed.

Determination of skeletal muscle GLUT4 protein concentration

Muscle samples were weighed frozen and then homogenized (VirTishear, Gardner, NY, USA) in HES buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 1 mM EDTA and 250 mM sucrose; pH 7.4; 1:20 wt vol^{-1}) using three 15-s bursts at high speed. The protein concentration of the homogenate was determined by the method of Bradford (1976). A 100 μL sample of the tissue homogenate was diluted 1:1 with the sample buffer as given in Laemmli (1970) (125 mM Tris base, 20% glycerol, 2% SDS, 0.008% bromophenol blue, 0.2 M dithiotheritol, pH 6.8)s and an aliquot of the diluted homogenate sample containing 75 μg of protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions on a 12.5% resolving gel for 1 h at a current of 50 mA on a Mini-Protean II dual slab cell (Bio-Rad, Richmond, CA, USA). Resolved proteins were transferred to polyvinylidene difluoride (PVDF) sheets (Bio-Rad, Richmond, CA, USA) by the method of Towbin *et al.* (1979) utilizing the buffer system of Kyhse-Anderson (1984), using a Bio-Rad SD semidry transfer unit at 150 mA for 11.5 min. After transfer, the PVDF sheets were rinsed in deionized water (2 × 10 min) and then stored overnight in deionized water at 4 °C. The following morning the PVDF membranes were blocked in Tween-Tris buffered saline (TTBS, 0.05% Tween-20) composed of 20 mM Tris, 500 mM NaCl (pH 7.5 at 25 °C) and 5% non-fat dry milk, pH 7.5. The sheets were washed in TTBS (1 × 5 min) and then washed (1 × 5 min) in a low salt Tween-Tris buffered saline (20 mM Tris, 0.9% NaCl) and 1% non-fat dry milk, pH 7.5. The membranes were then incubated for 1 h with the affinity purified polyclonal GLUT4 antibody R1184 A (donation of Dr Mike Gibbs, Pfizer Central Research, Groton, CN, USA) at a titre of 1:1500 in low salt TTBS and 1% milk. The PVDF sheets were then quickly rinsed in low salt TTBS, washed in TTBS for 10 min and washed in low salt TTBS for 10 min which was followed by incubation with HRP-labelled donkey anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL, USA) for 1 h at 37 °C. Next, the membranes were washed with sarcosyl buffer (3 × 20 min) at 37 °C to remove background activity and then rinsed in distilled H_2O (2 × 10 min). Antibody binding was visualized using enhanced chemiluminescence autoradiography in accordance with the manufacturer's instructions (ECL, Amersham Life Science, Arlington Heights, IL, USA). Labelled bands were quantified by capturing images of the autoradiographs in a Macintosh IIsi computer (Apple Computer Inc, Cupertino, CA, USA) with an image scanner (600 Plus Scanner, Mirror Technologies, Inc, St. Paul, MN, USA) equipped with a transparency module. The captured images were digitized and imported into imaging analysis software (NIH Image 1.55, Bethesda, MD, USA) and the density of the labelled 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 & Corkey 1969). Aliquots of the PCA extracts were stored at −80 °C until measured for ATP (Lamprecht & Trautschold 1974), creatine phosphate (Lamprecht *et al.* 1974), creatine (Bernt *et al.* 1974), and inorganic phosphate (Sigma Diagnostics, Kit #670-C, St. Louis, MO, USA).

The cytosolic phosphorylation state ratio, $[ATP][ADP]_f^{-1}[P_i]^{-1}$, was calculated from $[ATP][ADP]_f^{-1}[P_i]^{-1} = [CrP][Cr]^{-1}[P_i]^{-1} \times 1/K_{CPK}$, where $[ADP]_f$ is the calculated free cytosolic concentration of ADP and [ATP], [CrP], [Cr], and $[P_i]$ are the measured intracellular concentrations of ATP, creatine phosphate, creatine and inorganic phosphate, respectively. K_{CPK} is the pH-dependent equilibrium constant of creatine phosphokinase calculated according to Lawson & Veech (1979) at 1 mM free Mg^{2+} from the equation $\log K_{CPK} = 0.87\ pH + 8.31$.

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

Measurement of adenylate cyclase activity

Muscles were thawed over ice and finely minced in 4 mL of homogenization buffer (50 mM Tris, 5 mM EDTA and 90 mM NaCl, pH 7.5.) at 4 °C. The tissue was then homogenized, filtered through a layer of silk screen mesh to remove large pieces of connective tissue, and then 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^{-1}) in 1.5 mL of resuspension buffer. The final suspension was aliquoted and stored at −80 °C until analysed for adenylate cyclase activity and protein concentration (Bradford 1976).

For determination of adenylate cyclase activity a 100 μL aliquot of the membrane fraction was diluted in resuspension buffer to a final concentration of 0.30 mg protein mL^{-1} and added to a reaction mixture that contained 0.3 mM ATP, 100 μM GTP, 10 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, 1 mM 3-isobutyl-1-methylxanthine, 0.1% BSA, 25 U mL^{-1} creatine phosphokinase, 20 U mL^{-1} adenosine deaminase and 10 mM phosphocreatine along with one of the following stimulatory compounds: 100 μM forskolin, 5 mM NaF or 10 μM isoproterenol. The use of these stimulatory compounds allowed assessment of possible differences that may have existed in adenylate cyclase activity 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 (2000 × *g*). Duplicate 50 μL aliquots of the supernatant were removed for cAMP determination using a commercially available radioimmunoassay kit (Diagnostic Products Corp, Los Angeles, CA, USA). Adenylate cyclase activity is expressed as pmol cAMP mg $protein^{-1}$ min^{-1}.

Statistical analysis

The data were analysed using a two-way analysis of variance to test the effects of chronic low-frequency electrical stimulation and diet (treatment × time). Differences between means were determined using Newman–Kuels *post-hoc* test. Differences were considered significant if *P*-values less than 0.05 were obtained. All values are expressed as means ± SE.

RESULTS

Body weight, muscle weight and total muscle protein

The body weights of the β-GPA animals were significantly lower at each time point when compared to those of the normal diet animals (Table 1). These differences in body weight could not be attributed solely to differences in food consumption. Rats in the β-GPA diet group consumed ≈24 g of food per day while the normal diet rats consumed ≈26 g of food per day. Within dietary treatment groups, body weight did not differ over the stimulation period.

The weight of the non-stimulated control gastrocnemius muscle was significantly lower in the β-GPA diet group when compared to that of the normal diet group (Table 1). However, the gastrocnemius muscle weight did not differ between dietary groups at 14 and 28 days of electrical stimulation. Within groups, the weight of the gastrocnemius was significantly lower than the non-stimulated control muscle at 14 and 28 days of stimulation.

Skeletal muscle GLUT4 protein concentration

The β-GPA diet, in the absence of electrical stimulation, significantly increased skeletal muscle GLUT4 protein concentration by 60% (Fig. 1). Following 14 days of electrical stimulation, the GLUT4 protein concentration of the gastrocnemius was elevated above non-stimulated control muscles in both diet groups with the β-GPA diet continuing to exhibit a significantly greater GLUT4 protein concentration compared to the normal diet animals (99.3 ± 5.5% of heart standard vs. 65.3 ± 8.4% of heart standard). By 28 days of electrical stimulation, GLUT4 protein concentration did not differ between dietary groups. This was because of the GLUT4 protein concentration increasing in the normal diet group from day 14 of stimulation but not in the β-GPA diet group.

Adenylate cyclase activity and tissue cAMP concentration

Following 14 and 28 days of electrical stimulation, adenylate cyclase activity in the gastrocnemius was

Table 1 Body weight and gastrocnemius weight from female Sprague-Dawley rats subjected to various durations of continuous (24 h day^{-1}) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve while consuming either a normal or 2% β-GPA diet

	Normal diet			2% β-GPA diet		
	Control (7)	14 day (7)	28 days (9)	Control (7)	14 days (9)	28 days (9)
Body weight, g	252.1 ± 6.5	245.0 ± 3.9	250.0 ± 4.5	227.6$^{@}$ ± 4.8	220.6$^{@}$ ± 6.3	231.7$^{@}$ ± 4.2
Muscle weight, g	1.510 ± 0.07	0.881* ± 0.16	0.616* ± 0.08	1.161$^{@}$ ± 0.04	0.691* ± 0.09	0.604* ± 0.09

Values are means ± SE. Numbers in parentheses represent number of animals in each group. *, significantly different from control value of group ($P < 0.05$); $^{@}$, significantly different from the normal diet value at the corresponding time point ($P < 0.05$).

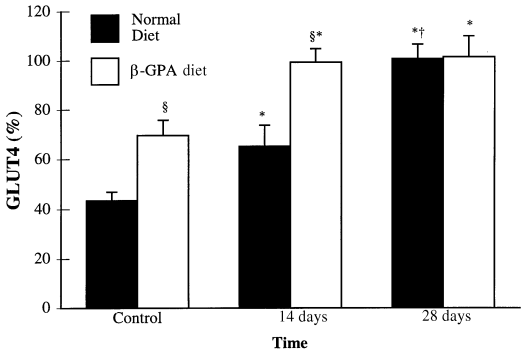

Figure 1 GLUT4 protein concentration, expressed as a percentage of a heart standard, in gastrocnemius muscle from female Sprague-Dawley rats subjected to various durations of continuous (24 h day^{-1}) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve while consuming either a normal or 2% β-GPA diet. Values are means ± SE. *, significantly different from the control value of the group ($P < 0.05$); †, significantly different from 14-day value of the group ($P < 0.05$); §, significantly different from the normal diet value at the corresponding time point ($P < 0.05$).

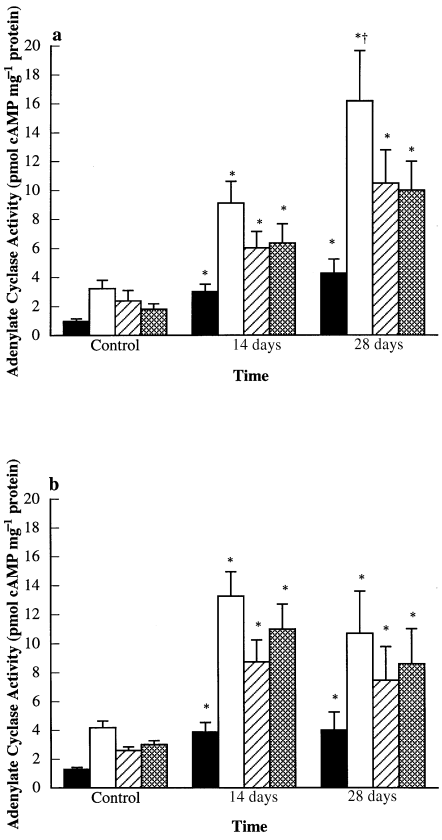

Figure 2 Adenylate cyclase activity (pmol cAMP mg^{-1} min^{-1}) in gastrocnemius muscle from female Sprague-Dawley rats subjected to various durations of continuous (24 h day^{-1}) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve while consuming either (a) a normal diet (■) GTP, normal diet, (□) GTP + forskolin, normal diet, (▨) GTP NaF normal diet, (▩) GTP + isoproterenol, normal diet; or (b) a 2% β-GPA diet (■) GTP, β-GPA diet, (□) GTP + forskolin, β-GPA diet, (▨) GTP+NaF, β-GPA diet, (▩) GTP + isoproterenol, β-GPA diet. Values are means ± SE. *, significantly different from control value of the group ($P < 0.05$); †, significantly different from the 14-day value of the group ($P < 0.05$).

significantly higher than in the non-stimulated control muscle in both diet groups, regardless of the stimulatory reagent utilized to assess activity (Fig. 2a,b). Within the normal diet group, it was observed that forskolin-stimulated adenylate cyclase activity at 28 days of electrical stimulation was significantly greater than at 14 days (Fig. 2a). No difference in adenylate cyclase activity existed between the β-GPA and normal diet groups.

Cyclic AMP concentration in the non-stimulated gastrocnemius was not statistically different between dietary treatment groups (Fig. 3). Following 14 days of electrical stimulation, cAMP concentration was significantly elevated above the non-stimulated control muscle in both dietary treatment groups. Additionally, it was found that at 14 days of electrical stimulation the skeletal muscle cAMP concentration of the β-GPA diet group was greater than that of the normal diet group (689.3 ± 64.2 vs. 517.3 ± 61.5 pmol g wet wt^{-1}). By 28 days of electrical stimulation, muscle cAMP concentration continued to be significantly elevated above that of the non-stimulated muscles, but no difference existed between dietary treatment groups.

Skeletal muscle high energy phosphate levels

The concentration of ATP, creatine phosphate, creatine and inorganic phosphate were significantly lower in the β-GPA diet group when compared to the normal diet group at all time points (Table 2).

In the β-GPA diet group, 14 days of electrical stimulation further reduced the concentration of ATP, creatine phosphate and creatine and then these variables remained at this reduced level throughout the 28 days of electrical stimulation. Creatine phosphate concentration was found to be below the detection sensitivity of the assay at 28 days of stimulation in the β-GPA diet group.

Following 14 and 28 days of electrical stimulation, the concentration of the high energy phosphate compounds declined below that of the non-stimulated control muscle in the normal diet group. By 28 days of stimulation, the concentration of ATP, creatine phosphate and inorganic phosphate was further reduced below that of muscles stimulated for 14 days.

DISCUSSION

A limited amount of information is available to indicate how an increase in skeletal muscle contractile activity translates into alterations in the physiological make-up

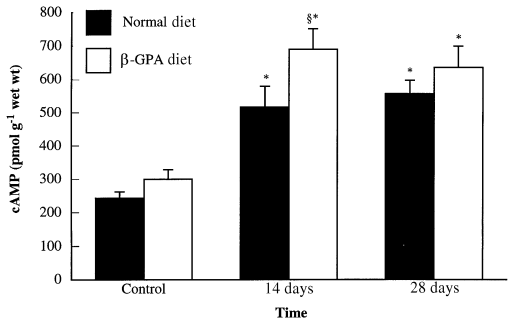

Figure 3 Cyclic AMP (cAMP) concentration in the gastrocnemius muscle from female Sprague-Dawley rats subjected to various durations of continuous (24 h day^{-1}) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve while consuming either a normal or 2% β-GPA diet. Values are means ± SE. *, significantly different from the control value of the group ($P < 0.05$); @, significantly different from the normal diet at the corresponding time point ($P < 0.05$).

of the activated muscle. Several intracellular signalling pathways have been associated with the changes that occur in skeletal muscle following an increase in skeletal muscle contractile activity, and thus have been suggested as initiators of the 'contraction signal'. Two pathways that have been considered to be likely candidates to generate the contraction signal are the β-adrenergic receptor–adenylate cyclase–cAMP pathway (Williams *et al.* 1984, Kraus *et al.* 1992) and alterations in the high energy phosphate state (Shoubridge *et al.* 1985, Lai & Booth 1990, Green *et al.* 1992, Ren *et al.* 1993). Recently, we investigated whether one or both of these pathways may mediate the contraction-induced increase in skeletal muscle GLUT4 protein concentration (Yaspelkis *et al.* 1997). It was found that both the adenylate cyclase–cAMP pathway and the high energy phosphate state of the muscle exhibited temporal relationships to elevations in skeletal muscle GLUT4 protein concentration in response to chronic low-frequency electrical stimulation. These findings suggested that both pathways may be a part of the intracellular signal that regulates GLUT4 protein concentration. However, as both of the proposed signalling pathways were related to the increased skeletal muscle GLUT4 protein concentration, the relative importance of the individual pathways could not be determined. Thus, the aim of the present investigation was to elucidate whether alterations in the high energy phosphate state of the skeletal muscle initiates the contraction signal independent of alterations in the adenylate cyclase–cAMP pathway.

Our first course of investigation consisted of manipulating the high energy phosphate state of the muscle in the absence of an increase in skeletal muscle contractile activity by providing our rats with a diet containing 2% β-GPA. It has previously been observed that Wistar rats which consumed a 1% β-GPA diet for 10 weeks exhibited a 90% decline in skeletal muscle creatine phosphate levels and a 50% reduction in ATP concentration (Fitch *et al.* 1975). Moreover, rats provided with a 1% β-GPA diet for 6–10 weeks have been found to have an increase in their skeletal muscle oxidative capacity that is similar to that which occurs with exercise training (Shoubridge *et al.* 1985, Lai & Booth 1990, Ren *et al.* 1993). As a β-GPA diet increases the oxidative capacity of skeletal muscle and as the GLUT4 protein concentration of the muscle is positively correlated with its oxidative capacity (Henriksen *et al.* 1990, Banks *et al.* 1992), it could be assumed that a β-GPA diet would also elevate the skeletal muscle GLUT4 protein concentration. This hypothesis was recently addressed by Ren *et al.* (1993) who administered a 1% β-GPA diet to Wistar rats for 6 weeks and found that plantaris and epitrochlearis GLUT4 protein concentration was substantially elevated by ≈50% above those from rats receiving a control diet. In agreement with the

Table 2 Adenosine triphosphate (ATP), creatine phosphate (CP), inorganic phosphate (P_i) concentration, calculated free adenosine diphosphate (ADP_f) and phosphorylation potential in the gastrocnemius muscle from female Sprague-Dawley rats subjected to various durations of continuous (24 h day^{-1}) low-frequency (10 Hz) chronic electrical stimulation via the tibial nerve while consuming either a normal or 2% β-GPA diet

	Normal diet			2% β-GPA diet		
	Control (7)	14 days (7)	28 days (9)	Control (7)	14 days (9)	28 days (9)
ATP, μmol g^{-1} wet wt	6.16 ± 0.42	2.73* ± 0.48	0.84*† ± 0.14	3.18@ ± 0.51	0.39@* ± 0.22	0.29* ± 0.21
CP, μmol g^{-1} wet wt	22.28 ± 1.36	9.32* ± 2.28	5.15*† ± 0.76	1.23@ ± 0.09	0.10@* ± 0.05	n.d.
Creatine, μmol g^{-1} wet wt	6.83 ± 0.55	3.11* ± 0.64	2.28* ± 0.37	2.17@ ± 0.38	1.35@* ± 0.22	1.02@* ± 0.16
P_i, μmol g^{-1} wet wt	5.54 ± 0.34	3.05* ± 0.36	2.40*† ± 0.26	1.29@ ± 0.12	1.24@ ± 0.09	1.05@ ± 0.06
ADP_f, μmol g^{-1} wet wt	0.13 ± 0.01	0.07 ± 0.02	0.02 ± 0.01	0.41@ ± 0.15	0.04* ± 0.03	n.d.
[ATP] $[ADP]_f^{-1}$ $[P_i]^{-1}$, $\times 10^4$ M^{-1}	9.25 ± 0.88	14.73* ± 1.57	16.34* ± 2.34	8.14 ± 1.53	0.64@* ± 0.44	n.d.

Values are means ± SE. Numbers in parentheses represent the number of animals in each group. *, significantly different from the control value of group ($P < 0.05$); †, significantly different from the 14-day value of group ($P < 0.05$); @, significantly different from the normal diet value at the corresponding time point ($P < 0.05$). n.d., below detection sensitivity of the assay which resulted in the phosphorylation potential for this treatment group being incalculatable.

findings of Ren *et al.* (1993), we observed that providing rats with a diet that included 2% β-GPA significantly reduced the level of the skeletal muscle high energy phosphate compounds and increased muscle GLUT4 protein concentration by ≈60%.

Green *et al.* (1992) have suggested that alterations in the high energy phosphate state of the muscle during contraction reflects a central control element of cellular metabolism, and as such, initiates the contraction-induced adaptations in skeletal muscle. This hypothesis has recently been advanced by Wiesner (1997) who reviewed how alterations in cellular energy supply and demand may increase mitochondrial gene expression. It appears that disturbances in cellular energy metabolism such as changes in ATP/ADP, redox status or oxygen may directly act on mitochondrial transcriptional regulators such as mitochondrial transcription factor A resulting in an increased rate of mitochondrial biogenesis. The fact that elevations in muscle GLUT4 protein concentration are positively correlated with muscle oxidative capacity (Henriksen *et al.* 1990, Banks *et al.* 1992) suggests that increased expression of the GLUT4 protein may be similarly regulated. Several observations from the present investigation support the hypothesis that the contraction-induced elevations in skeletal muscle GLUT4 protein concentration resulted from alterations in cellular energy supply. The first was that skeletal muscle GLUT4 protein concentration was elevated in the normal diet rats following 14 days of electrical stimulation to a level similar to that of the non-stimulated β-GPA animals, and that ATP and creatine were reduced to the same level in both the normal diet animals that were stimulated for 14 days and in the β-GPA animals that received no stimulation. Second, the high energy phosphate compounds were substantially reduced following 14 days of stimulation in the β-GPA animals, but then did not further decline. This was similar to the pattern of change for muscle GLUT4 protein concentration in the β-GPA animals which peaked at 14 days and did not increase with additional days of stimulation. Third, the skeletal muscle high energy phosphate compounds of the normal diet group achieved their lowest concentration at 28 days of stimulation which also corresponded to a peak in their skeletal muscle GLUT4 protein concentration. Finally, it was found that elevations in skeletal muscle GLUT4 protein concentration were significantly correlated to the decline in ATP concentration in both the normal ($r = -0.73$, $P < 0.05$) and β-GPA ($r = -0.63$, $P < 0.05$) diet groups. Thus, these observations collectively suggest that alterations in the skeletal muscle high energy phosphate state may initiate the increase in muscle GLUT4 protein concentration.

The β-GPA diet animals exhibited a significantly greater muscle GLUT4 protein concentration at 14 days of electrical stimulation when compared to the normal diet group. The possibility exists that the greater GLUT4 protein concentration at 14 days of stimulation was a result of the combined effects of the β-GPA diet and electrical stimulation elevating cAMP levels to a greater extent than that which occurred with electrical stimulation alone. It has previously been reported by Kraus *et al.* (1992) that 21 days of electrical stimulation increases cAMP levels in rabbit tibialis anterior muscle and that the elevated cAMP levels are related to the increased oxidative capacity of the muscle. Direct support for the role of cAMP on gene expression has recently been provided by Osawa *et al.* (1995) who demonstrated that the cAMP analog, 8-(4-chlorophenyl-thio)-cAMP, increases hexokinase II gene expression in L6 cells which are a representative muscle cell line. This finding is of significance in that it has been observed that elevations in skeletal muscle hexokinase activity and GLUT4 protein concentration are coregulated in response to chronic electrical stimulation (Hofmann & Pette 1994, Kong *et al.* 1994) and exercise training (Ren *et al.* 1994).

Although elevated cAMP levels may facilitate the contraction signal, alterations in the β-receptor signalling pathway do not necessarily appear to be required for skeletal muscle GLUT4 concentration to be increased. This was clearly evident in the non-stimulated β-GPA animals in which it was observed that skeletal muscle GLUT4 protein concentration was elevated ≈60% above non-stimulated control diet animals despite no difference in muscle cAMP levels. Likewise, in the normal diet animals, skeletal muscle GLUT4 protein concentration increased between 14 and 28 days of electrical stimulation even though cAMP concentration did not increase over this time period. In addition, adenylate cyclase activity, as assessed by the various biochemical stimulatory compounds, was not different between dietary treatment groups at any time point during the experimental period. Therefore, these observations suggest that the elevations in skeletal muscle GLUT4 protein concentration cannot be accounted for solely by changes in the β-receptor signalling pathway. Rather, it is more likely that the β-adrenergic signalling pathway serves to potentiate the effects of another intracellular signal such as alterations in cellular energy supply.

The combined effects of 28 days of chronic electrical stimulation and β-GPA diet did not increase skeletal muscle GLUT4 protein concentration above that which occurred with stimulation alone. Rather, the combination of treatments served only to accelerate the normal time course of events suggesting that muscle contraction and β-GPA diet act on the same signalling pathway. Thus, the accelerated course of events resulted in the β-GPA animals achieving a peak increase

in skeletal muscle GLUT4 protein concentration by 14 days of stimulation whereas the normal diet animals did not approach their capacity to fully increase muscle GLUT4 protein concentration until 28 days of stimulation. This observation is in agreement with Etgen *et al.* (1993b) who reported that increases in skeletal muscle GLUT4 protein concentration begin to plateau by 30 days of chronic electrical stimulation.

In summary, skeletal muscle GLUT4 protein concentration was significantly elevated in animals that were provided with a 2% β-GPA diet in the absence of any rise in muscle cAMP levels or adenylate cyclase activity. The combination of a 2% β-GPA diet and 14 days of chronic electrical stimulation resulted in skeletal muscle GLUT4 protein concentration being greater compared to contraction alone. In conjunction with this greater rate of rise in GLUT4 protein concentration at 14 days of stimulation in the β-GPA animals, cAMP concentration was elevated and the high energy phosphate compounds exhibited a greater decline. Following 28 days of stimulation, skeletal muscle cAMP levels did not further increase and GLUT4 protein concentration was not different between dietary treatment groups. These findings suggest that the contraction-induced increase in skeletal muscle GLUT4 protein is initiated by alterations in cellular energy supply and not the β-adrenergic receptor signalling pathway. However, the results do not preclude the possibility that an increased intracellular cAMP concentration potentiates the effect of an altered high energy phosphate state which in turn may accelerate the rate of increase in skeletal muscle GLUT4 protein concentration.

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