Volume 310, number 2, 129–131 FEBS 11576 September 1992

Time-dependoent increases in Na^+,K^+-ATPase content of low-frequency-stimulated rabbit muscle

H.J. Green[a], M. Ball-Burnett[a], E.R. Chin[a], L. Dux[b,*] and D. Pette[b]

[a]*Department of Kinesiology, University of Waterloo, Waterloo, Ont. N2L 3G1, Canada* and [b]*Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Germany*

Received 5 August 1992

Chronic low-frequency stimulation of rabbit fast-twitch muscle induced time-dependent increases in the concentration of the sarcolemmal Na^+,K^+-ATPase and in mitochondrial citrate synthase activity. The almost twofold increase in Na^+,K^+-ATPase preceded the rise in citrate synthase and was complete after 10 days of stimulation. We suggest that the increase in Na^+,K^+-ATPase enhances resistance to fatigue of low-frequency-stimulated muscle prior to elevations in aerobic-oxidative capacity.

Low-frequency stimulation; Muscle fatigue; Na^+/K^+-ATPase; Sarcolemma

1. INTRODUCTION

Chronic low-frequency stimulation leads to a conversion of fast-twitch, fast-fatigable muscles into slow-twitch, fatigue-resistant muscles [1,2]. Fast-to-slow transitions in the expression of myofibrillar protein isoforms and Ca^{2+}-regulatory protein isoforms are accompanied by marked increases in aerobic-oxidative capacity, as reflected by elevated enzyme activities of the aerobic-oxidative metabolic pathways (citric acid cycle, fatty acid oxidation, respiratory chain). As generally assumed, these latter changes seem to be of major importance for enhanced resistance to fatigue, however, a time-course study on changes in resistance to fatigue and citrate synthase activity, a representative enzyme of aerobic-oxidative capacity, in low-frequency-stimulated fast-twitch muscles of rat and rabbit has cast doubt on the general validity of this notion [3]. Asynchronous changes of these two parameters, as well as increases in fatigue resistance prior to major increases in citrate synthase activity suggested the possibility that as yet unidentified factors might effect improved fatigue resistance, especially during the early period of chronic stimulation.

Thus, it appeared interesting to study the effect of chronic low-frequency stimulation on the sarcolemmal Na^+,K^+-ATPase which serves to re-establish Na^+/K^+ gradients following excitation. Circumstantial evidence suggests that the Na^+,K^+-pump activity is rate limiting under conditions of sustained contractile activity, and that its sarcolemmal concentration is insufficient for maintaining the excitability of the post-synaptic membrane [4,5]. Therefore, we have examined whether and to what extent chronic low-frequency stimulation affects the concentration of the Na^+,K^+-ATPase in rabbit fast-twitch muscle. For this purpose, we used the same animals as in our previous study on changes in metabolites of energy metabolism at various time points between 15 min and 50 days after the onset of stimulation [6]. Na^+,K^+-ATPase was assessed by the [^{3}H]ouabain binding method [7]. This method is based on the fact that ouabain binds specifically to the Na^+,K^+-ATPase and that this binding is saturable, reversible and stoichiometric [8]. In order to compare the time course of changes in Na^+,K^+-ATPase with stimulation-induced increases in aerobic-oxidative capacity, measurements of citrate synthase activity were performed in the same muscles.

Correspondence address: D. Pette, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany. Fax: (49) (7531) 883 940.

**Present address:* Albert Szent-Györgyi University, Medical School, Department of Biochemistry, PO Box 415, Szeged, Hungary.

2. MATERIALS AND METHODS

2.1. *Animals and chronic stimulation*

Adult male and female White New Zealand rabbits weighing between 2.5 and 4.2 kg were implanted with electrodes and the left peroneal nerve was stimulated continuously (24 h/day) by telestimulation up to 50 days. Stimulation was at 10 Hz, 0.15 ms pulse width, with supramaximal voltages (range 1.6–5 V) individually set for each animal [9]. Nine different stimulation periods were selected for investigation. These included 15 min, 60 min, 3 h, 12 h, 24 h, 2 days, 4 days, 10 days and 50 days. Three to four animals were examined at each time point. The non-stimulated right extensor digitorum longus (EDL) muscles served as controls. Additional control EDL muscles were obtained from animals that had been implanted with electrodes, but were not stimulated. Following each stimulation period, animals were

anesthetized and the right, non-stimulated and left, stimulated EDL muscles dissected. The muscles were rapidly frozen in liquid N_2 and stored at −70°C until analysed.

2.2. *Measurement of Na^+,K^+-ATPase concentration*

Na^+,K^+-ATPase concentration was assessed by the vanadate-facilitated, stoichiometric binding of [^{3}H]ouabain to small muscle fragments [7,10]. Frozen muscle samples were cut into small pieces (3–6 for each sample) weighing 2–8 mg and washed 2 times for 10 min in a buffer (pH 7.3) containing 10 mM Tris-HCl, 3 mM $MgSO_4$, 1 mM Tris-vanadate, and 250 mM sucrose. Samples were then incubated 2 times for 60 min at 37°C in the same buffer with the addition of [^{3}H]ouabain (2 μCi/ml) and unlabeled ouabain (1 μM final concentration). Unbound ouabain was removed by washing 4 times for 30 min in ice-cold buffer (see above). Thereafter, the samples were blotted, weighed, placed in 1.5 ml Eppendorf tubes and soaked in 1 ml 5% trichloroacetic acid for 16 h at room temperature before counting for ^{3}H radioactivity in a scintillation mixture. The [^{3}H]ouabain binding capacity was expressed in pmol per g wet weight. All values were corrected for non-specific uptake and the retention of [^{3}H]ouabain (determined in the presence of unlabeled ouabain), as well as for the loss of [^{3}H]ouabain during the washing procedure. The factor to adjust for [^{3}H]ouabain wash-out was 1.05 [7].

2.3. *Citrate synthase activity*

Citrate synthase was measured fluorometrically in muscle homogenates (1:100 dilution) prepared in 0.17 M phosphate (pH 7.4), 0.02% bovine serum albumin, 5 mM 2-mercaptoethanol. The assay mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM malate, 0.5 mM NAD and 18 U/ml malate dehydrogenase. The reaction was started by the addition of acetyl-CoA (0.22 mM final concentration).

2.4. *Statistical analyses*

Statistical analyses of the data for both the Na^+,K^+-ATPase and the citrate synthase was accomplished using a one way ANOVA procedure for time. Where significant differences were found, Newman-Keul's post hoc procedures were used to identify significantly different means. At least a 95% confidence level ($P < 0.05$) was set for all comparisons.

3. RESULTS

Since there was no statistical evidence of effects of age on both Na^+,K^+-ATPase concentration and citrate synthase activity, all control values for the non-stimulated EDL muscles were combined. The Na^+,K^+-ATPase concentration ($\bar{X} \pm$ S.E.M., $n = 48$) for all controls amounted to 248 ± 8. The corresponding value for citrate synthase activity was 4.31 ± 0.2 μmol × min^{-1} × g^{-1} wet weight.

Chronic low-frequency stimulation had pronounced effects on both Na^+,K^+-ATPase concentration and citrate synthase activity. Significant increases ($P < 0.05$) in Na^+,K^+-ATPase concentration were first observed after 4 days of stimulation (Fig. 1). This increase continued, reaching a value of 461 ± 67 pmol/g wet weight after 10 days. As compared to the controls, this corresponded to an 86% increase. Throughout the remaining period of stimulation, no further increase was observed and the Na^+,K^+-ATPase concentration amounted to 466 ± 5 pmol/g wet weight after 50 days of stimulation.

Large increases were found in citrate synthase activity (Fig. 1), however, as compared to Na^+,K^+-ATPase, this increase occurred later. Significant increases

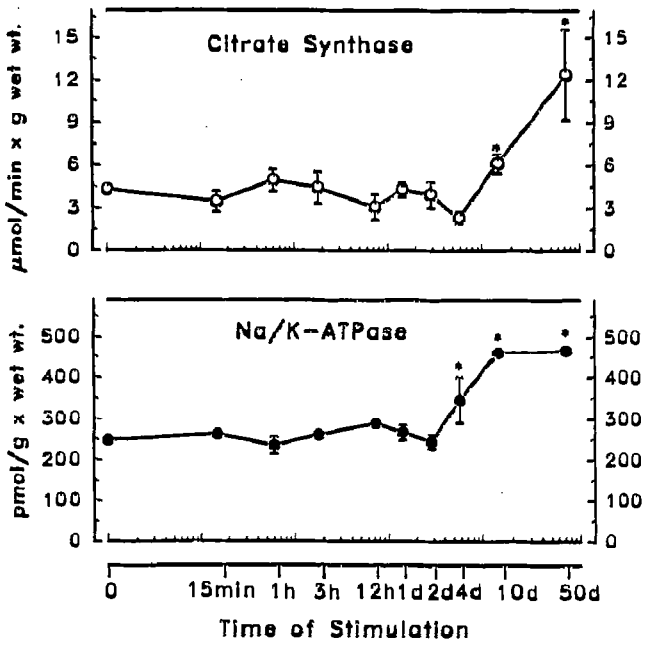

Fig. 1. Time-course of changes in citrate synthase activity and in the concentration of sarcolemmal Na^+,K^+-ATPase as induced by chronic low-frequency stimulation in fast-twitch extensor digitorum longus muscle of rabbit. Values are means ± S.E.M. $n = 48$ for zero time and 3–4 animals for each time point of stimulation. Asterisks mark significant ($P < 0.05$) difference from zero time control.

($P<0.05$) in citrate synthase activity were not detected until 10 days of stimulation. Unlike the Na^+,K^+-ATPase, stimulation beyond 10 days produced further elevations in citrate synthase activity. By 50 days of stimulation, citrate synthase activity had reached an approximately 3-fold higher level than that in the unstimulated control muscles (12.4 ± 3.2 μmol × min^{-1} × g^{-1} wet weight).

4. DISCUSSION

The present results agree with previous observations on training-induced increases in the concentration of ouabain-binding sites in muscles from rat and human. Kjeldsen and collaborators [11] reported up to 46% increases in Na^+,K^+-ATPase concentration of rat fast-twitch extensor digitorum longus and slow-twitch soleus muscles following 6 weeks of an intense swim training. Conversely, inactivity led to decreases in ouabain binding [11]. Training-induced increases in the concentration of Na^+,K^+-pumps were also found in human muscle [12,13]. That the observed increase in Na^+,K^+-ATPase concentration of rabbit fast-twitch muscle (approximately 90%) is markedly higher than with various exercise training regimens may be explained by the higher amount of contractile activity that can be imposed by chronic low-frequency stimulation.

Exercise is associated with a net loss of K^+ from the working muscle [14–19]. Under conditions of intense

contractile activity, the capacity of the sarcolemmal Na^+,K^+-ATPase may become a limiting factor, such that potassium efflux may exceed the capacity for potassium re-accumulation [4]. Potassium imbalance may impair sarcolemmal excitability and consequently muscle performance [5]. In fact, chronic low-frequency stimulation of rabbit fast-twitch muscle has been shown to result in an approximately 50% reduction of isometric force within the first 5 min after the onset of stimulation [6]. It may be suggested that this early fatigue reaction relates to the loss of K^+ because of an insufficient K^+ re-accumulation in the unconditioned muscle. The pronounced increase in Na^+,K^+-ATPase concentration observed after only 4 days represents an early adaptive response, serving to meet the functional demands of increased contractile activity.

Another relevant result of this study relates to the time course of the stimulation-induced changes. The rise in Na^+,K^+-ATPase clearly precedes the increase in citrate synthase. As judged from the time points studied, the increase in Na^+,K^+-ATPase is complete after 10 days, whereas citrate synthase continues to rise thereafter, as previously confirmed in several studies [2]. Taken together, the time courses of these changes clearly point to the functional significance of elevated Na^+,K^+-ATPase concentrations as an early adaptive response which contributes to enhanced resistance to fatigue in low-frequency stimulated muscle.

Acknowledgements: Supported by the National Science and Engineering Research Council (NSERC) of Canada and Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 156. L.D. thanks the Alexander von Humboldt Foundation for a stipend.

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