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CREATINE AND ACTIN IN REGENERATING RAT GASTROCNEMIUS MUSCLES.

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ABSTRACT

Gastrocnemius muscles of 150 g rats were minced and orthotopically autografted. The regenerates were examined 15 to 240 days after the surgery. Actin concentrations increased continuously, albeit at a rate decreasing as the regeneration proceeded. Total creatine increased in proportion to actin. The maximum tetanic tension of the regenerate increased linearly with actin. Creatine-kinase activity increased as a curvilinear function of total creatine.

INTRODUCTION

Most of the studies in muscle energetics were aimed at identifying the chemical reactions that set free the energy needed for the work and heat produced by a contracting muscle, and at describing their time course. These studies have been very successful, leading in turn to the discovery of oxidations, glycolysis and the Lohmann reaction[1]. The energy balance studies in which the energy set free by the chemical reactions is compared to the energy (heat + work) produced by the muscle, have shown that except for some potentially important details, these chemical reactions account for the energy flow in the contracting muscle[2-3].

The quantitative description of the enzymes responsible for the energy flow in muscle has led to the concept of constant proportion groups of enzymes as well as constant ratios of key enzymes representing various metabolic system ('system relations')[4]. Enzymic activities of single fibers of the same histochemical fast or slow type may vary within very wide limits[5] but not if the fibres belong to the same motor unit[6].

Little is known on the mechanism that control the long term adaptation of the enzymes to the physiological requirements. There seems to be a good reason for this situation. Whereas it is very easy to trigger a very large increase in energy flow by stimulating a muscle, it is more difficult to modify its enzy-

matic equipment[7]. It seems therefore that enzymes in muscles are controlled by well protected homeostatic mechanisms We think that the study of the enzymes adaptation might be easier if is limited to that of a very simple system submitted to a perturbation as large as possible.

The system that we have chosen for our study is the Lohmann reaction: it is composed of only four substrates, creatine (Cr), phosphocreatine (PCr), ATP and ADP, and one enzyme creatinekinase (CPK).

The Lohmann reaction offers a second advantage. Energetic substrates such as glycogen, lactate or O_2, vary within wide limits according to the physiological state of the muscle. It would be futile to seek quantitative relations between these substrates and the amount of contractile proteins. ATP and ADP are more constant, but their ubiquitous roles makes the interpretation of changes in their concentration rather difficult.

The situation is very different for the sum (creatine + phosphocreatine) = total creatine, C_T. The concentration of C_T is remarkably constant. It does not depend of the physiological states of rest or fatigue [8,9]. Its concentration measured in the striated muscles of many animals, from human to frog, stay within a quite narrow range (15 to 30 µmoles per gran fresh weight. In frog sartorius muscle, C_T is strongly correlated with the tension (force/unit area) developed by the muscle, or with the amount of energy used during a contraction [8,9]..Carlson (1963) and Wilkie (1968), discussing this property, have suggested the use of C_T concentration as a measure of muscle "size". In their view, muscle "size" ca be measured by any extensive parameter that is proportional to its maximum tetanic force (for parallel muscle such as sartorius) or its heat rate production during a maximal isometric tetanus ("stable maintenance heat" of Aubert (1956). The amount of contractile proteins is another example such as actin.

We expect therefore to find proportional relationships between C_T, CPK, actin concentration and tension production.

The perturbation that we have choosen is the mincing of the muscle. The ensuing regeneration of the muscle can be thought of as a recovery from a large perturbation of the stationary equilibrium which normally exists in muscle.

We have found that actin concentration increases considerably during the muscle regeneration. Total creatine increases in proportion to actin, but tension and creatinekinase increase in different ways.

RESULTS

a. Correlation between total creatine and actin

In a first series of experiments thirty right triceps surae of female Wistar rats weighing about 150 g were removed, cut into very small pieces with scissors and grafted back into the animal's leg, big tendons and soleus being eliminated from the mince[10]. The regenerated muscles were obtained 15, 30, 60, 120 and 240 days later (n = 6 for each duration). The left muscles were used as control. The muscles were extracted[11] with 0.1 M KCl, 0.001 M TES. Creatine was measured in the supernatant by the α-naphtol diacetyl method, in duplicate.

Actin was measured in the pellet by the following method. The precipitate was dissolved in 1 mM SDS and electrophoresed[12] on a slab gel. The gel was stained with Coomassie blue. Its optical density was measured with an automated scanner. The surface area of the actin peaks was obtained by numerical integration. Pure actin was treated in the same way. Integrated peak areas of actin were proportional to the actin deposited on the gel. The calibration curve thus obtained was used to compute the amount of actin in the sample gel. Each sample was electrophoresed in duplicate, one aliquot being exactly twice the other one. In this way it could be ascertained that the amount of actin measured on the gel was proportional to the amount of protein deposited on the gel. The actin concentration was then computed per gram wet weight of muscle.

The actin concentration measured in 30 control gastrocnemius muscles was equal to 27 $\pm$ 3 % of the sedimentable myofibrillar proteins, 17 $\pm$ 2 % of the total proteins or 0.49 $\pm$ 0.05 µmole

per gram wet weight muscles. These estimates are in agreement with those found in the litterature[13-14-15].

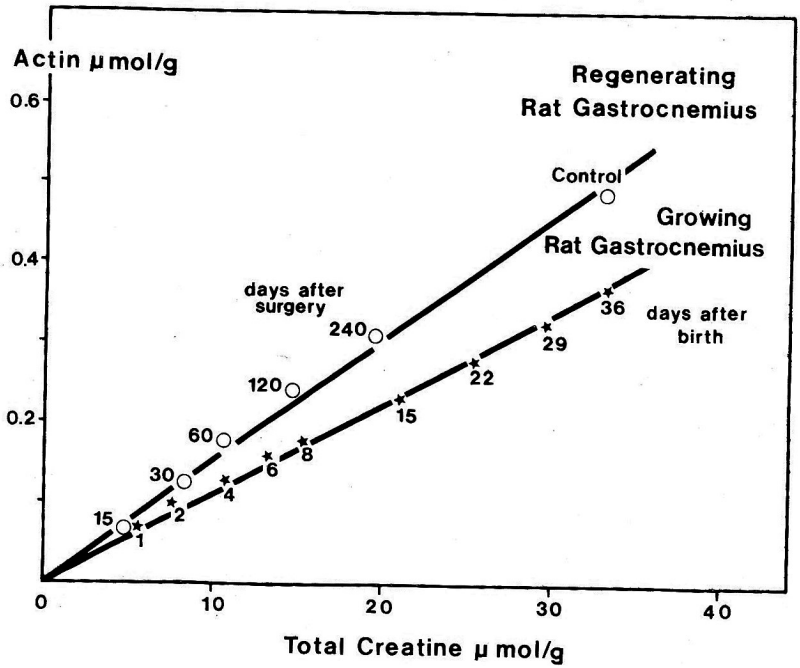

Figure 1

Figure 1 summarises the results obtained in regenerating muscles. Each point (hollow circles) is the mean of 4 to 6 experiments. The standard deviations of the means (not shown) are about equal to the diameter of the circles for C_T, and twice that diameter for actin. Actin and total creatine concentrations have been measured in the same muscles. The number near the points indicate the duration of regeneration in days after the surgery. Actin and total creatine increase rapidly in the early phases of regeneration, and more slowly thereafter, as is shown by the fact that the distance between the points for 15 and 30 days is about the same than that between the points for 120 and 240 days. The actin concentration remains proportional to the total creatine concentration at all stages of the regeneration. The slope of this

relation is equal to 0.016 ± 0.002 μmole actin/μmole C_T. However, the regeneration is far from being complete even 240 days after the surgery, as actin and total creatine are then about 2/3 of the normal value. If the control point is included in the regression, the slope becomes 0.015 ± 0.002. This slope is not statistically different from the one calculated when the control point is ommitted.

If the proportionality between actin and total creatine concentration is of any deep significance, it should be found in another situation in which there occurs a large increase in total creatine i.e. in post-natal growth. This hypothesis is checked in fig. 1 by the experimental points shown by stars. These points have been determined as follows. Actin concentrations were taken from Uchino and Tsuboi (1970)[16] for the various post-natal days shown as parameters. Total creatine concentrations were estimated according to Maréchal and Plaghki (1979) who reported that in growing rat gastrocnemius[17] : $C_T = 1.33 \sqrt{t}$ (C_T in μmoles/g, t in postnatal days). Although the estimates were obtained from two experiments widely separated in space and in time, the actin concentration is proportional to the total creatine concentration with a slope of 0.011 ± 0.002. This slope is not much different from that obtained for the case of regenerating gastrocnemius. Thus, the main conclusion that emerges from these experiments is that actin concentrations are proportional to total creatine concentrations in regenerating as well as in post-natally growing muscles.

b. Correlation between actin and mechanical tension

In a second series of experiments we have measured the maximum isometric force of the regenerating muscles. The regenerate was carefully dissected, leaving intact its vascular and nervous supply, as well as its proximal tendon. The distal tendon was tied to an electromagnetic ergometer[18]. The sciatic nerve was exposed in the thigh, and its collaterals were cut under binocular. The sciatic nerve was stimulated by alternating condenser discharges, and an isometric force-length curve was obtained. These experiments were performed on regenerates aged 15, 30, 60 or 120 days. The developed tension was computed by dividing the maximal force (in Newton) by the wet weight of the muscle and

multiplying the result by the muscle length. The maximal isometric tension of regenerates of various ages is plotted in fig. 2 as a function of the actin concentration as measured in the experiments referred to in section a. The relationship is linear, but not proportional. The slope of the line is equal to 572 ± 16 kN/m^2 per µmole of actin. The intercept on the abscissae may mean that no tension can be generated if the actin concentration is below

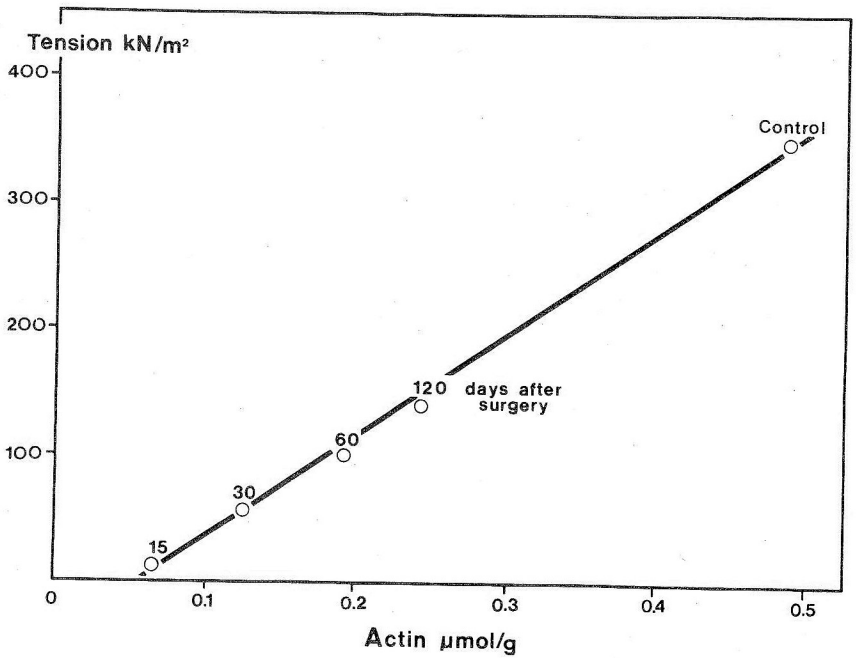

Figure 2

0.05 µmole/g. It may also indicate the existence of an actin pool in muscle that is not directly concerned in the production of tension. A third possibility is that it reflects the progressive reinnervation of the regenerates[19] : as the regenerates were indirectly stimulated, the non-innervated muscle fibres would not contribute to the developed tension, whilst their actin content would be taken into account. We are of the opinion that this last possibility is the most likely, because we found that the younger the regenerate, the greater the force produced by direct stimulation relative to that resulting from an indirect stimula-

tion. However, we have as yet not enough data to check quantitatively this hypothesis.

c. Correlation between total creatine and creatinekinase

The activity of creatinekinase was measured[20] together with total creatine in the muscle extracts of the experiments described in a. Figure 3 shows how this activity changes when the total creatine increases during the regeneration of gastrocnemius muscle. Each point is the mean of 6 experiments. The standard errors of the mean (not shown) are about equal to the diameter of the circles. It is obvious that these parameters do not grow proportionally.

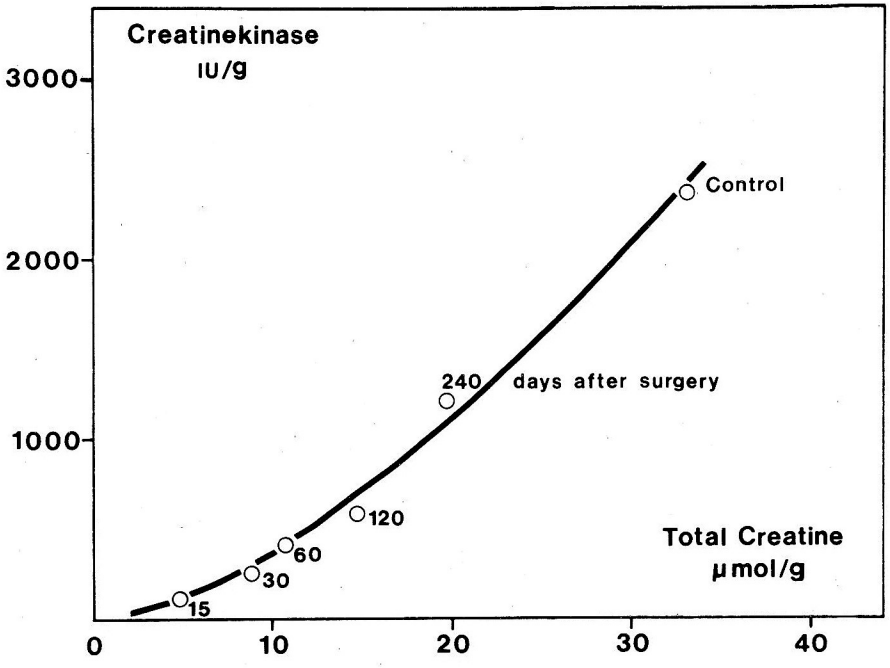

Figure 3

A standard statistical analysis of the data confirmed that the relation was not linear. It was analyzed according to a least rectangle procedure which yielded the power function : (creatinekinase) = 10.7 (total creatine)$^{1.55}$. The function is shown in fig. 3 by the solid line.

DISCUSSION

We have studied the adaptation of two components of the Lohmann reaction to a strong perturbation. Gastrocnemius muscles of adult rats were minced, orthotopically autografted and examined at various times after the surgery during their regeneration.

ATP and ADP concentrations were not measured in this study, as it is known that their concentration is very variable in regenerating frog muscles, showing no obvious relationship to total creatine[21]. Free creatine was not measured either. Its concentration depends critically on the physiological status of the muscle[1-2]; therefore, it must be measured in resting muscle, a condition that is not easy to obtain. It is known that in regenerating frog muscles at rest, it remains at a constant ratio to the total creatine[21].

Total creatine increases during the regeneration. It shows two remarkable relationships. On the one hand its increase is strictly proportional to that of actin but on the other hand creatinekinase increases at a rate that is faster than that of total creatine[11].

The proportional relationship between total creatine and actin is found in growing as well as in regenerating muscle, there being about 70 to 80 molecules of creatine (phosphorylated or not) to every molecule of actin. If the synthesis of actin is stimulated by the average dosage of creatine in regenerates as it is the case in myoblasts cultures[22], the fact of a constant proportion of total creatine vs actin may indicate that total creatine is a measure of the maximum possible signal that controls the actin synthesis. In this view the synthesis of actin would be controlled not by the mean free creatine dosage (which depends in turn of the mean amount of exercice performed by the muscle) but by the ratio of the free creatine to the total creatine.

It has been reported that various proteins of the contractile apparatus increase in regenerating muscle in proportion to actin[23]. Thus it may be that total creatine may not only control the synthesis of actin, but perhaps the coordinated syntheses of all the contractile proteins of the sarcomere as well.

It is then tempting to speculate that if total creatine play such a central role in the control of the contractile apparatus, it is to be expected that its concentration must be fixed by very

efficient homeostatic mechanism. This would account for the fact that total creatine concentration is quite constant in all striated muscles. In this view regeneration would occur by successive addition of elementary units of contraction, sarcomeric proteins and total creatine, the control of the process being obtained via the mean free creatine level, itself dependent on the functional requirements of the animal. As long as the regenerate is too small, its fibres would have to contract often and strongly, thereby maintaining a large mean concentration of free creatine that would in turn stimulate the syntheses of actin and of other proteins. The tension would then be proportional to total creatine or actin content. This is indeed what is found if it is considered that the findings shown in fig. 2 should be corrected for the fact that the reinnervation of muscle fiber is progressive and requires at least 60 to 90 days to be complete[19].

However, things are not that simple. A disturbing fact is that creatinekinase activity does not increase in proportion to total creatine when the muscle regenerates (fig. 3). We have no explanation to offer for this. We must however point out that the case is not an isolated one. Several enzymes of the glycolytic system increase their activities at various rates, during muscle regeneration[11]; in that respect they do not obey the 'constant proportion theory'[4].

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