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F. Guba, G. Maréchal, O. Takács (eds)

CREATINE, CREATINEKINASE AND GLYCOLYTIC ENZYMES IN REGENERATING RAT CASTROCNEMIUS

L. Plaghki, M. Colson-Van Schoor, G. Beckers-Bleukx and G. Maréchal
Laboratoire Physiologie Générale, UCL-5540, 8-1200 Brussels, Belgium

Abstract

Rat gastrocnemius muscles were minced and orthotopically autografted. They were analyzed 1, 3, 6, 15, 30, 60, 120 and 240 days after the surgery for the activities of creatinekinase, glycogen phosphorylase, phosphofructokinase, aldolase, pyruvatekinase and lactic dehydrogenase. These enzymes remain in a constant proportion, but only as a first approximation. Their percentual relative rates of increase remain invariant throughout the regeneration.

Introduction

Comparative analyses have shown that the activities of a number of enzymes vary within wide limits in various muscles of different animals but that enzymes of the same pathway remain in a constant proportion to each other (1). Well documented examples have been presented for the enzymes of the glycolysis (2). However some enzymes of the glycolytic pathway do not change in proportions during postnatal growth of rat (3) or pig (4) muscle,

It is therefore of interest to investigate whether the "constant proportion" theory holds in the case of muscle regenerating in an adult animal. We have used the model described by Carlson (5). According to him, minced gastrocnemius muscles orthotopically autografted regenerate within 2 to 3 weeks. His histologic studies suggest that regeneration largely recapitulates ontogeny.

It is shown in this work that the molar ratios of creatinekinase and of five enzymes of the glycogenolytic-glycolytic pathway remain in a constant proportion during muscle regeneration in adult animal, but only as a first approximation. The work reveals that the parameters which remain invariant during the regeneration are the percentual relative rates of increases of the enzyme activities.

These relative rates of the enzymes must be assessed against a reference that should be a good index of muscle "size". We have chosen total creatine, C_T (sum of phosphorylcreatine and free creatine), for this purpose because a) total creatine is strongly correlated with the force and the heat produced by a contracting muscle (6, 7), and b) it is proportional to actin both in growing rat muscles (8-9) and in regenerating rat muscles (9).

Methods

Animals.

Forty-eight Wistar rats weighing approximately 150 g were used for these experiments. The right triceps surae was excised, minced and orthotopically autografted after removal of the soleus muscle. No post-operative complications have been observed (5).

Muscle extracts.

The regenerates and the left gastrocnemius (used as control) were extracted in 2 ml of ice-cold 80 mM KC1, 1 mM TES (pH 7.3) 0.1 mM dithiothreitol, homogenized at 0°C first with an Ultraturrax mixer (thrice 10 s at full speed) then with a Potter Evelghem apparatus. The Potter apparatus *was* washed twice with 2 ml of the extracting solution, and the wash was added to the extract. The extract was centrifuged 20 min at 2,600 g at 0°C. The precipitate was washed twice with 2 ml of the extracting solution and the three supernatants were combined. The precipitate was suspended in 1 mM TES (1 ml for 100 mg muscle).

Chemical analyses.

Total creatine was measured by the a-naphtol diacetyl method. Creatinekinase, glycogen phosphorylase, phosphofructokinase, aldolase and lactic dehydrogenase were assayed by the methods described in Bergmeyer (10). The results are expressed in International Units (at 25°C) per gram of wet weight muscle (g).

The analyses were performed on the supernatants and on the suspended precipitates. The precipitates contained less than 1% of the total creatine and less than 3%'of the enzymic activities. There was one exception, phosphofructokinase. Its activity in the precipitate amounted to about 60% of that found in the supernatant. All the activities and concentrations reported in this paper are the total activities (supernatant + precipitate).

Results

The results are shown in Table 1. The regenerates were excised 1, **3.** 6, 15, 30, 60, 120 and 240 days after the surgery. Six animals were used for each duration of regeneration, making 48 animals for the whole experiment. The eight means obtained for the control values at each post-operative period were statistically equal; therefore the 48 control values were pooled and their mean is presented in Table 1.

Table 1 shows clearly the two phases that follow the surgery. In the first one that extends over the days 1 to 6, the enzyme activities and the creatine contents are very low. This phase corresponds to the histologic degeneration (5). In the second one, all the parameters increase. This phase corresponds to the histologic regeneration of muscle fibres (5).

Table 1

Days post surgery	n	Total Creatine µmol/g	Creatine kinase IU//g	Phospho-rylase IU/g	Phospho-fructo kinase IU/g	Aldolase IU/g	Pyruvate kinase IU/g	Lactic dehydro-genase IU/g
1	6	0.8 ±0.05	88 ±13	0.7 ±0.2	10.5 ±2.8	1.5 ±0.2	11 ±1	42.9 ±6.0
3	6	1.3 ±0.2	40 ±12	0.8 ±0.3	4.3 ±0.7	2.2 ±0.4	9 ±2	42.8 ±4.3
6	6	1.1 ±0.2	27 ±11	0.4 ±0.1	5.3 ±1.3	1.5 ±0.3	14 ±2	49.5 ±10.0
15	6	4.3 ±0.9	123 ±46	2.2 ±0.9	8.7 ±1.2	4.1 ±1.3	20 ±2	67.8 ±10.0
30	6	8.2 ±0.8	265 ±49	4.4 ±1.5	11.9 ±2.5	5.9 ±1.4	27 ±3	91.0 ±10.5
60	6	10.6 ±1.4	424 ±63	9.7 ±1.3	14.7 ±2.2	12.4 ±1.7	42 ±4	131.2 ±13.1
120	6	14.1 ±1.3	580 ±41	16.7 ±1.6	17.4 ±3.2	18.5 ±2.4	58 ±6	232.7 ±17.2
240	6	18.6 ±0.8	1500 ±132	28.7 ±3.3	22.9 ±2.1	35.1. ±3.9	109 ±8	399.9 ±25.4
Control	48	33.1 ±0.4	2461 ±74	61.1 ±1.9	36.1 ±2.2	61.2 ±1.5	178 ±8	484.1 ±15.4

It must be observed however that even 240 days post-surgery, the biochemical parameters are still about 60% of the control values. This is the case for total creatine (59%), phosphofructokinase (63%) and aldolase (56%). Creatinekinase and glycogen phosphorylase have recovered somewhat less, 45 and 48% respectively, whilst pyruvatekinase and lactic dehydrogenase have recovered somewhat more, 66 and 69% respectively.

Discussion

Pette (2) has discussed the theory that the enzymes of a common path-way e.g. glycolysis remain in a constant molar ratio. Accordingly the first point to discuss is whether these activities of the five enzymes of the glycolytic pathway estimated in this study, remain in a constant molar ratio during the regeneration.

The molar ratios for the control rat gastrocnemius are shown in Table 2, assuming for the enzymes molecular weights and specific activities (in IU per mg muscle) identical to those reported by Pette (2). Phosphofructokinase has been chosen as the reference enzyme. The ratios are close to those reported by Pette (2) for rabbit abductor magnus, except for glycogen phosphorylase which is five times more abundant than expected.

Table 2

Enzymes	Specific activities IU/mg (a)	Molecular weight kdaltons (a)	Molar ratio (b)	Molar ratio (c) in regenerating muscles — days post surgery: Control	15	30	60	120	240
Glycogen Phosphorylas	12,6	185	122	62	10	14	25	37	48
Phosphofructokinase	240	373	1	1	1	1	1	1	1
Aldolase	23	158	24	42	12	12	21	26	37
Pyruvate kinase	445	237	5	4	2	2	2	3	4
Lactic dehydrogenase	574	140	11	14	9	9	10	15	15

(a) reported by Pette (2); (b) computed from Table 3 in Pette (2); (c) computed from the data of Table 1 using the specific activities and molecular weights reported in reference (2).

Table 2 shows that during regeneration the molar ratios remain practically constant only for two enzymes, namely, pyruvate kinase and lactic-dehydrogenase. The molar ratios of glycogen phosphorylase and of aldolase increase steadily during the regeneration. The variations in molar ratio are however moderate, not exceeding three to five times. Thus the theory of constant proportion holds in the case of regenerating muscles, but only as a first approximation.

Another method of analysis is the following. If the enzymes remain in a constant proportion during the regeneration, then the regression of their activities to that of phosphofructokinase should be linear with a zero intercept. The data of days 1, 3 and 6 after the surgery have been excluded from the calculations, as it is clear that by then the regeneration has not yet started.

The results of these calculations are shown in Table 3. The intercept A is statistically different from zero for glycogen phosphorylase, aldolase and pyruvate kinase. The regression coefficients are highly significant. Therefore the enzyme activities are described by a linear but not proportional function of phosphofructokinase.

Table 3

	Glycogen phosphorylase	Aldolase	Pyruvate kinase	Lactic dehydrogenase
b	2.17	2.21	5.99	15.3
S_b	±0.10	±0.11	±0.58	±1.3
a	-20.2	-18.3	-38.3	-68.6
S_a	±2.1	±2.3	±12.0	±27.1
t_4	9.48	8.08	3.19	2.53
	0.001	0.002	0.05	0.1
r	0.99	0.99	0.98	0.98

The data of Table 1 are analyzed according to the regression:

(Activity of enzyme) = a + b (activity of phosphofructokinase)

S_b and S_a are the standard errors of b and a. t4 equals the ratio a/S_a. P indicates the probability that a is different from zero. r is the correlation coefficient between the activity of the enzyme and that of phosphofructokinase.

These results suggest that the enzymes regenerate at different rates during the regeneration. We have chosen total creatine as a standard against which to assess their rates because total creatine is a good index of muscle "size" (cfr. Introduction).

The data of Table 1 were analyzed to the following concept. The activity (E) of an enzyme increases at a procentual rate that is equal to dE/E.dt; at the same time, the procentual rate of increase of total creatine concentration is $dC_T/C_T.dt$. Our specific hypothesis is that:

$$\{dE/E.dt\} / \{dC_T/C_T.dt\} = \boldsymbol{C} \qquad (1)$$

Integration of equation (1) gives:

$$E = B \,.\, (C_T)^{\boldsymbol{C}} \qquad (2)$$

Equation (2) is fitted to the data of Table 1 (excluding days 1, 3, 6) by a least rectangle analysis. The results of the computation are shown in Table 4.

Table 4

	Creatine kinase	Glycogen phosphorylase	Phospho fructo-kinase	Aldolase	Pyruvate kinase	Lactic dehydrogenase
B	12.3	0.17	2.90	0.43	3.15	13.2
C	1.52	1.69	0.70	1.42	1.14	1.03
r	0.98	0.99	0.99	0.98	0.97	0.97
C_1	1.31	1.41	0.58	1.08	0.82	0.77
C_2	1.67	2.03	0.85	1.87	1.59	1.37

Rectangular regression analysis of enzyme activities (E) and total creatine concentrations (C_T) of Table 1 according to the equation (2. r: correlation coefficient between E and C_T. C1 and C2 are the fiducial limits of ***C*** at a probability level of 5%, computed from the equations $C1 = \boldsymbol{C}\,(1+k/2-\sqrt{k})$ and $C2 = \boldsymbol{C}\,(1+k+\sqrt{k})$ when $k = t^2(1 - r^2) / (n-2)$. $t_4 = 2.776$ (Student "t"; 4 d.f. < 5%.

If C = 1, the activity of the enzyme is proportional to the total creatine concentration. This is the case for pyruvatekinase and lactic dehydrogenase. Aldolase and glycogen phosphorylase increase at relative rates at are respectively 69% and 42% higher than that of total creatine. On the other hand, phosphofructokinase increases at a rate that is only 70% that of total creatine. It is obvious that the five glycolytic enzymes tested increase at rates that are constant during the regeneration, but at are different for each enzyme. Their absolute amount cannot therefore remain in a constant proportion, a conclusion reached already above.

Creatinekinase also increases at a relative rate that is different from that of total creatine, being higher by 52%. The difference in rate is very significant from a statistical viewpoint. The fiducial limits for the constant C = 1.52 are 1.21 and 1.82 at the probability level of 1% and 1.02 1 2.16 at the probability level of 1%.

It seems that one conclusion emerges from these experiments. Growth of generating muscle does not occur by a simple addition of small physiological units of constant composition. This does not mean that the various components that make up the muscle cells increase during regeneration more or less at random. To the contrary, they increase in a coordinate pattern is means that something remains constant. The invariant parameter is not a proportion of the components, their increases not being proportional each other. The invariant parameter is their relative percentual rate of increase, i.e., at any instant the percentual rate of increase of the component is a constant fraction of the percentual rate of increase of any other component. This property results directly from the fact that equation (1) describes all the data.

If the percentual rates of increase are equal for two components, the two components grow in proportion to each other. This is the case for total creatine, actin (9), tension (9) and various proteins of the contractile apparatus (11). It is not the case for various enzymes that control the energy flow in muscle (cfr. Table 4). Their relative proportions change with the size of the regenerates (as measured by total creatine), a process similar to that discussed by Gould (12) in his review the subject of allometry in ontogeny and phylogeny.

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