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PROGRESSIVE LOSS OF MITOCHONDRIAL CREATINE PHOSPHOKINASE ACTIVITY IN MUSCULAR DYSTROPHY

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SUMMARY: Mitochondria were isolated from the pectoralis and gastrocnemius muscles of chickens with a hereditary muscular dystrophy, and age-matched controls. In the pectoralis, for dystrophic birds aged 0.12, 0.25, 0.55, and 1.55 yr, the creatine phosphokinase activity of the intact mitochondria, expressed in terms of pellet protein, was 69%, 45%, 24%, and 13% as great, respectively, as that of the controls. The corresponding figures for the gastrocnemius were 79%, 46%, 51%, and 28%. The mitochondria from dystrophic muscles exhibited satisfactory respiratory control ratios, P:O ratios, and state 3 respiratory rates. To check whether their apparent loss of creatine phosphokinase activity was due to the presence of increasing amounts of non-mitochondrial pellet protein, the state 3 respiratory rate was used as a mitochondrial marker; the rates per mg protein were similar in mitochondria from normal and dystrophic muscles of each age group.

INTRODUCTION

After a step-like or impulse-like change in the rate of ATP hydrolysis by a skeletal muscle, its rate of oxygen consumption changes with a time course provocatively similar to the response of a first order system (1-3). Moreover, the rate of oxygen consumption apparently changes in parallel with the concentration of creatine (2, 4-6). We have therefore hypothesized (5,6) that respiration in skeletal muscle is normally rate-limited by the mitochondrial creatine phosphokinase reaction. This prediction adds quantitative detail to a popular model (7-15; cf. Fig. 1).

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Abbreviations: Cr, creatine; CP, creatine phosphate; CPK, creatine phosphokinase; EGTA, ethylene glycol bis (β aminoethyl ether) N,N, N'-tetraacetic acid.

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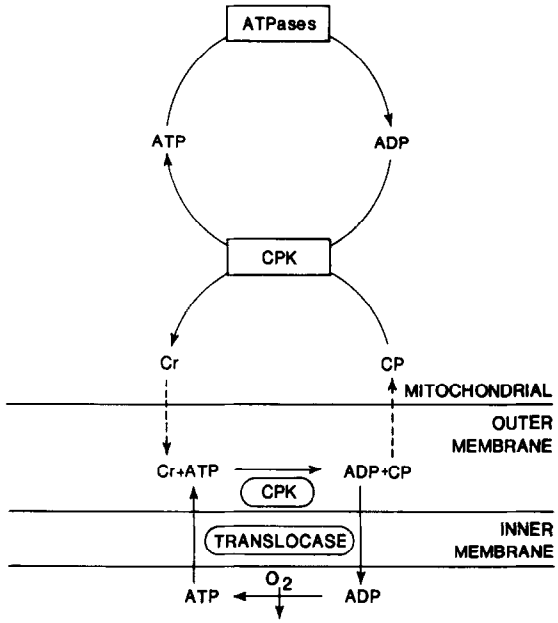

Figure 1. Schematic diagram showing a hypothesized role of mitochondrial CPK in the control of respiration in muscle.

In normal skeletal muscle at an early embryonic stage, extramitochondrial CPK activity resides entirely in the BB isoenzyme.[1] BB activity then decreases and finally disappears; simultaneously, MB activity increases, then decreases and disappears, or nearly so; and MM activity, after a relatively late appearance, gradually increases to its adult level. By birth or shortly thereafter, extramitochondrial CPK activity usually resides entirely in the MM isoenzyme. At earlier stages of this transition, the active isoenzymes can be the BB and MB, the BB, MB, and MM, or the MB and MM forms (19-28). In the muscular dystrophies of several species, including man, the pattern of extramitochondrial CPK isoenzyme activities in adult skeletal muscle is similar to that in normal fetal muscle: in addition to a high level of MM activity, appreciable MB activity, and in some cases low levels of BB activity, are evident (21, 29-33). It is not known whether the CPK profile regresses to this fetal-like state, or never leaves it. Within this context, an observation made by Hall and DeLuca (34)

1. Extramitochondrial CPK is a dimer, and two different types of subunit exist, the so-called brain (B) and muscle (M) types. These combine to form the BB, MB, and MM isoenzymes (16-18).

has an added significance: they reported that in cardiac muscle of the mouse and rabbit, mitochondrial CPK activity is absent until several days after birth. This suggests that the mitochondrial isoenzyme of CPK might be absent in fetal skeletal muscle as well. If so, and if the similarity between the CPK profiles of fetal and dystrophic muscle extends to the mitochondrial isoenzyme, one would expect this enzyme to be absent, or progressively lost, in muscular dystrophy. The experiments described here were designed to investigate this possibility.

MATERIALS AND METHODS

The Department of Avian Sciences of the University of California at Davis furnished chickens from a line with hereditary muscular dystrophy (line 455) and a paired control line (#454). White Leghorn chickens obtained locally were also used as controls. For all quantitites measured, the values obtained from these control groups were not significantly different, and have therefore been pooled. The pectoralis and gastrocnemius muscles were homogenized in an ice-cold medium containing 0.35 M mannitol, 10 mM Tris-phosphate (pH 7.2), 0.1 mM EGTA, and 50,000 I.U./ℓ heparin (35,36). Muscles were minced with scissors, put through a tissue press with 1.0 mm holes, weighed, then homogenized by hand in a Dounce-type vessle, using 4-9g tissue per 60 ml of homogenizing medium, and pestles with clearances of 0.30 mm, then 0.20 mm. The homogenate was centrifuged for 10 min at 500g. The supernatant was filtered through three layers of cheesecloth, then centrifuged for 10 min at 8000g. The whitish layer adhering to the mitochondrial pellets was removed by swirling, and the pellets were washed with homogenizing medium, combined, resuspended gently in a small glass homogenizer with Teflon pestle, centrifuged for 10 min at 8000g, and suspended in 0.7-1.5 ml of a medium with the same composition as the homogenizing medium, but without heparin and with 5g/100 ml dialyzed bovine serum albumin.

CPK activity in the intact mitochondria was assayed in the reverse direction (ADP + CP → ATP + Cr) at 30°C and pH 7.0, by coupling the CPK reaction to those catalyzed by hexokinase and glucose-6-phosphate dehydrogenase with Calbiochem kits, and measuring the increase in NADPH absorbance at 340 nm with a Gilford recording spectrophotometer. For mitochondrial protein contents of 1-10 µg/ml, the absorbance change was always linear with time, and for a given preparation, the reaction rate was proportional to the mitochondrial protein content. When CP was omitted from the medium, the absorbance change was negligible.

After solubilization for 30 min at 95°C in 0.9 N NaOH, mitochondrial protein was measured by the method of Lowry et al. (37), using bovine serum albumin as a standard. Spot checks with the method of Bradford (38) gave similar results.

Mitochondrial oxygen consumption at 30°C was measured in a Gilson Oxygraph (Model KM) with Clark oxygen electrode, using a medium containing 0.25 M mannitol, 10 mM KCl, 10 mM Tris-HCl (pH 7.2), and 0.1 mM EDTA. During an experiment, the following compounds were added, with the indicated final concentration: 5 mM potassium phosphate buffer (pH 7.2), 3.5 mM α-ketoglutarate, 0.3 mM ADP, and 1.4 mm $MgCl_2$.

In order to solubilize mitochondrial CPK, mitochondrial suspensions were diluted with a medium containing 0.1 M sodium phosphate buffer (pH 8), 10 mM beta mercaptoethanol, and 1 mM EDTA, and incubated overnight. Electrophoresis and visualization of the CPK isoenzymes in this extract were done by the methods of

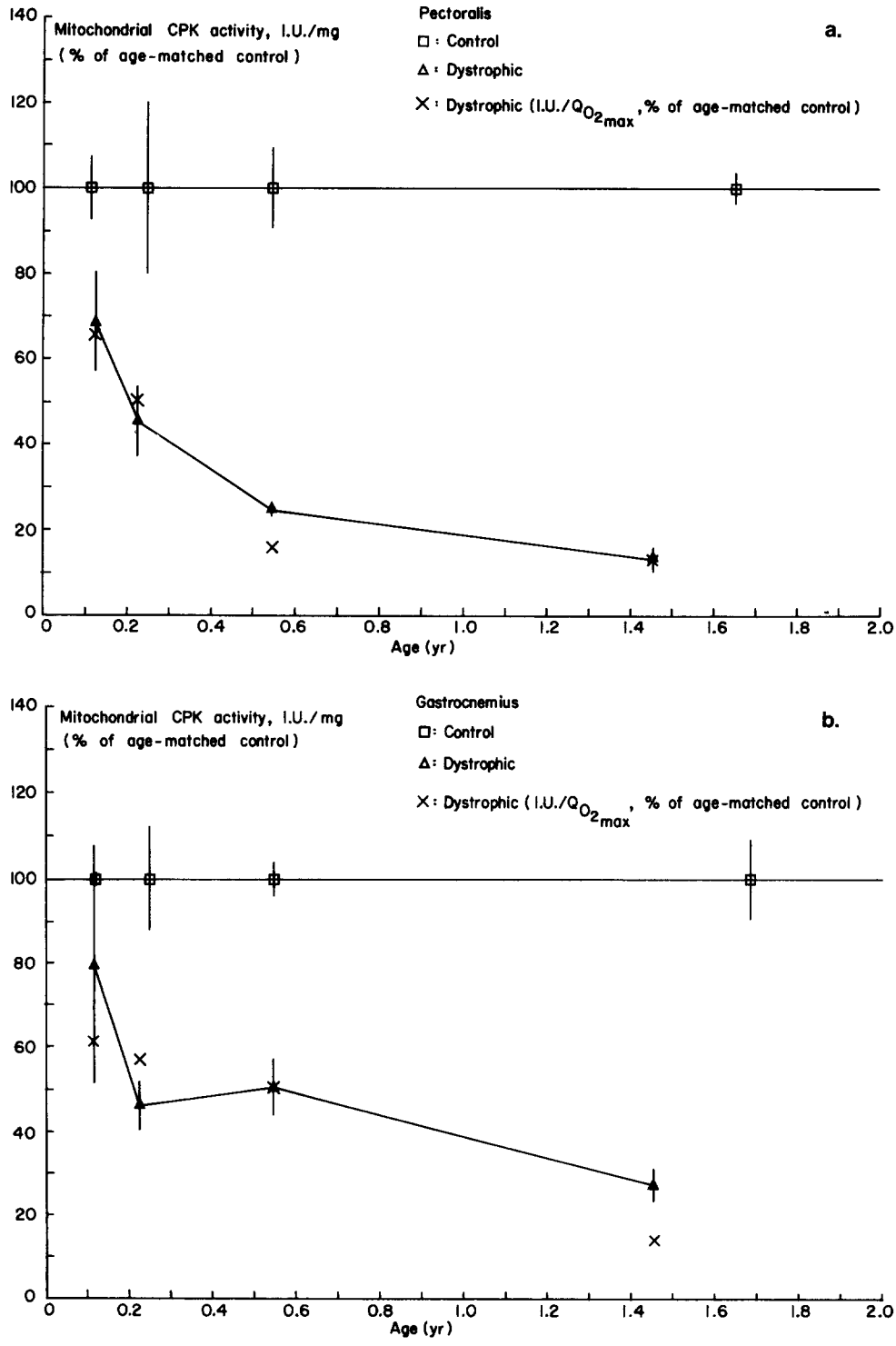

Figure 2. Mitochondrial CPK activity during the course of muscular dystrophy. □ , Δ : CPK activity expressed in terms of mitochondrial protein content; X: CPK activity expressed in terms of state 3 respiratory rate. Bars denote ± S.E.M.

Hall et al. (39). To quantify the migration of the MM isoenzyme, electrophoresis was done on a muscle homogenate prepared as described above, and centrifuged for 10 min at 500g.

RESULTS AND DISCUSSION

The CPK activities of mitochondria isolated from normal and dystrophic muscles of increasing age are given in Table 1 and Fig. 2. They suggest that, as hypothesized, a large decrease in mitochondrial CPK activity occurs during the course of muscular dystrophy. As shown in Table 2 and Fig. 3, for each age group the P:O ratio, respiratory control ratio, and state 3 respiratory rate of the mitochondria from the dystrophic muscles were similar to those of the controls, and consistent with literature values. Similar results have been reported for a variety of species (41-47). Even in occasional preparations lacking in respiratory control, from both normal and dystrophic muscles, the CPK activities were not markedly different from those of the corresponding preparations which did exhibit satisfactory respiratory control. These results suggest that the progressive decline in CPK activity of the mitochondria from dystrophic muscles was not simply part of a general decline in the integrity of the mitochondria, either *in vivo* or as isolated.

Dystrophic muscle is known to contain increasing amounts of degenerating structures of various types; it thus seemed possible that the pellets prepared from these muscles might have contained progressively larger amounts of non-mitochondrial protein, and that this might have accounted for the observed

TABLE 1. CPK activity of mitochondria from normal and dystrophic muscle

	CPK activity (I.U./mg mitochondrial protein)			
	Pectoralis		Gastrocnemius	
Age (yr)	Normal	Dystrophic	Normal	Dystrophic
0.12	13.9±1.0(4)[a]	9.6±1.6(4)	14.5±0.2(2)	11.6±4.1(4)
0.25	16.4±3.3(5)	7.5±1.4(3)	13.8±1.7(5)	6.4±0.8(3)
0.55	28.2±2.7(2)	7.1±0.3(2)	20.7±0.8(2)	10.5±1.4(2)
1.55	18.1±0.7(2)	2.4±0.5(5)	14.9±1.4(3)	4.1±0.6(4)

[a] mean±S.E.M.(n)

TABLE 2. Respiration in mitochondria from normal and dystrophic muscle

	Respiratory control ratio		P:O ratio		Maximal respiratory rate[a]	
	Pectoralis		Pectoralis		Pectoralis	
Age(yr)	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
0.12	3.9±0.6(4)[b]	2.1±0.4(4)	4.0±0.4(3)	3.1±0.4(4)	281±52(4)	268±42(4)
0.25	2.9±0.3(9)	3.5±0.4(2)	3.6±0.1(9)	3.6±0.2(2)	231±37(9)	255±61(3)
0.55	3.7±0.9(2)	3.0±0.4(2)	3.3±0.4(2)	3.2±0.5(2)	234±6 (2)	359±24(2)
1.55	3.3±0.5(2)	2.5±0.2(2)	3.2±0.1(2)	3.7±0.0(2)	240±26(2)	188±14(3)
	Gastrocnemius		Gastrocnemius		Gastrocnemius	
	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
0.12	4.4±1.4(2)	2.1±0.4(5)	3.7±0.3(2)	3.1±0.3(5)	307±64(2)	364±91(4)
0.25	2.4±0.3(5)	3.5±0.3(2)	3.1±0.2(5)	4.0±0.3(2)	347±54(6)	284±55(3)
0.55	2.7±0.3(2)	4.0±0.4(2)	2.4±0.2(2)	3.5±0.4(2)	322±20(2)	320±56(2)
1.55	2.4±0.4(3)	2.5±0.3(3)	3.5±0.7(3)	3.7±0.0(3)	162±44(3)	299±21(3)

[a] μatom O/min·mg protein [b] mean±S.E.M.(n)

decrease in CPK activity per mg pellet protein from the dystrophic muscles. To test this possibility, we used the state 3 respiratory rate of the preparations as a mitochondrial marker, on the assumption that it would be determined entirely, or at least nearly so, by intact mitochondria. As shown in Table 2, the state 3 respiratory rates per mg pellet protein were not consistently different in the suspensions prepared from normal and dystrophic muscles, indicating that there were probably also no important differences in the amounts of non-mitochondrial protein. Fig. 2 shows that the pattern of CPK activity in the mitochondria from dystrophic muscle, relative to that of the controls, is essentially the same whether the CPK activity is expressed in terms of the absolute state 3 respiratory rate or the amount of pellet protein.

With increasing age, the dystrophic muscles contained large, increasing amounts of fat (cf. 40). Mitochondrial CPK activity has so far been found only in muscle and nervous tissues (10,48), and although no direct evidence appears to be available, presumably does not occur in adipose cells (15). It was possible

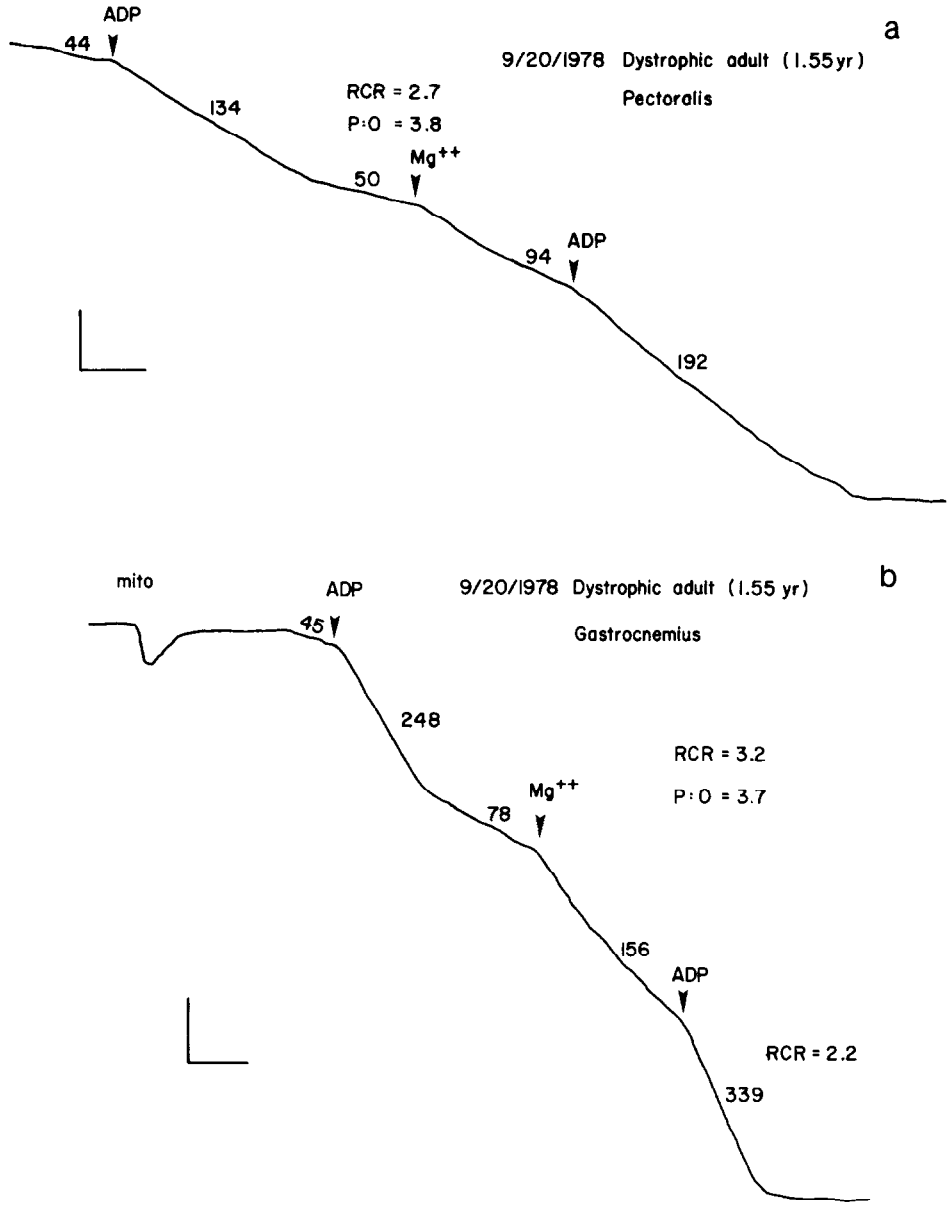

Figure 3. Oxygen electrode traces obtained with suspensions of mitochondria from a dystrophic chicken aged 1.55 yr. Arrows denote additions of ADP and Mg^{++}. Numbers above traces are respiratory rates, in μatom O/min · mg. Vertical and horizontal calibration bars designate 60 μatom O and 30 sec, respectively.

that the pellets isolated from the dystrophic muscles contained a substantial, increasing fraction of mitochondria from fat cells, and that this accounted at least in part for the observed decrease in CPK activity. To test this possibility,

50g of fat was processed by the method used to isolate mitochondria from muscle. This produced a small, diffuse, brownish pellet for which the protein yield per g of tissue was less than 10% of that for muscle; when this pellet was suspended in the usual way, its respiratory rate in the absence of added ADP was very low, and did not increase after the addition of 0.3 mM ADP. We conclude that the pellets obtained from dystrophic muscle did not contain significant amounts of fat mitochondria.

There appear to be no previously published values for the CPK activity of mitochondria from chicken skeletal muscle, but the values obtained here for normal muscles, about 15 I.U./mg mitochondrial protein, are substantially higher than those reported by other workers for mammalian skeletal and cardiac muscle, which are for the most part in the range 1-3 I.U./mg (10,11,48,50). To determine whether this discrepancy was species-dependent, we applied our methods to skeletal and cardiac muscle of the rabbit and rat, with the results summarized in Table 3. The differences between these values and those previously reported are apparently not due simply to differences in assay conditions[2]. Artefactually high values for mitochondrial CPK activity could have resulted if, during the isolation procedure, extramitochondrial (MM) CPK became associated with the mitochondria. We thus considered it possible that our results were influenced by the association of appreciable amounts of the MM isoenzyme with mitochondria from normal muscles, but of progressively smaller amounts with the mitochondria

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2. Mitochondrial CPK of rat heart and skeletal muscle exhibits rapid equilibrium random kinetics (10,11,13,50). From the general rate equation (13), it follows that in the absence of products,

$$v/V_{max} = xy/(1+x+y+xy),$$

where x denotes $[S_1]/K$, y denotes $[S_2]/K_2$, and K_1 and K_2 are the dissociation constants (K_m's) for S_1 and S_2. The K_m's for CP and ADP are 4.0 mM and 0.06 mM, respectively, for rat skeletal muscle (50), and 0.50-0.72 mM and 0.035-0.051 mM, respectively, for rat heart (10,13). The concentrations of CP and ADP in the reverse direction CPK assay used in the present study were 20 mM and 1.2 mM, respectively, from which it can be calculated that v/V_{max} was about 0.79 in rat skeletal muscle mitochondria, and 0.93-0.95 in rat heart mitochondria. The corresponding values calculated for the previous studies listed in Table III are roughtly similar. All of the measurements appear to have been made at 30°C, and at a pH near 7.

TABLE 3. Survey of reported values for mitochondrial CPK activity

Tissue	Reference	CPK_{mito} (I.U./mg)
chicken pectoralis	This study	17.5±2.0(13)[a]
chicken gastrocnemius	This study	15.2±1.0(12)
rabbit skeletal muscle	This study	5.6±0.5(4)
rabbit heart	Sobel et al. (1972)	2.9
	This study	4.5±0.6(4)
rat skeletal muscle	Jacobs et al. (1964)	1.0
	Jacobus and Lehninger (1973)	2.4
	Saks et al. (1977)	2.7±0.5
	This study	8.0±0.2(6)
rat heart	Jacobs et al. (1964)	1.0
	Jacobus and Lehninger (1973)	1.6
	Saks et al. (1973)	2.8-4.8
	This study	6.8±0.4(3)

[a] mean±S.E.M.(n)

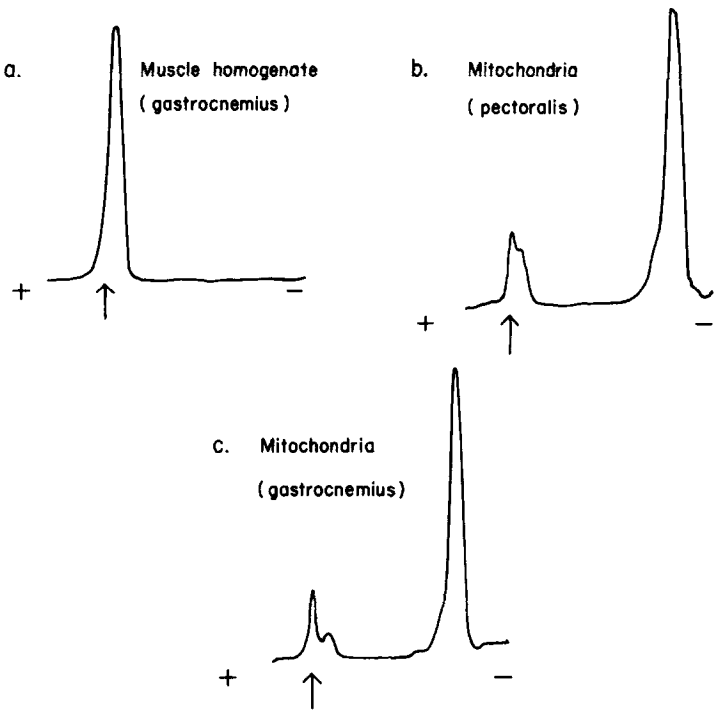

Figure 4. Electrophoretic analysis of CPK from a whole-muscle homogenate (a) and mitochondrial extracts (b,c). Arrow indicates origin.

TABLE 4. Mitochondrial protein yields

Tissue	Mitochondrial protein yield (mg/g tissue)			
	Age			
	0.12 yr	0.25 yr	0.55 yr	1.55 yr
pectoralis, normal	0.12±0.01(4)[a]	0.22±0.05(9)	0.12±0.01(2)	0.13±0.06(2)
pectoralis, dystrophic	0.32±0.02(4)	0.38±0.14(3)	0.17±0.01(2)	0.10±0.02(5)
gastrocnemius, normal	0.35±0.18(2)	0.23±0.04(6)	0.32±0.07(2)	0.16±0.04(3)
gastrocnemius, dystrophic	0.29±0.04(4)	0.27±0.07(3)	0.30±0.02(2)	0.19±0.02(5)
rat skeletal muscle	0.52±0.06(6)			
rabbit skeletal muscle	0.53±0.07(4)			
rat heart	8.2±1.0(3)			

[a] mean±S.E.M.(n)

from the dystrophic muscles. However, two lines of evidence argued against this possibility. First, additional rounds of washing, suspension, and centrifugation did not lessen the CPK activity of mitochondria from normal muscle. Second, as shown in Fig. 4, electrophoresis of mitochondrial extracts established that almost all the CPK of mitochondria isolated from normal muscles migrated as the mitochondrial isoenzyme, and only a small fraction as MM. It appears that the high mitochondrial CPK activities reported here are genuine. The lower mitochondrial CPK values reported by others for normal muscle might be due to differences in isolation procedures. With the method used here, cells were disrupted by a hand homogenization which was presumably relatively gentle. Mitochondrial protein yields (cf. Table 4) were considerably lower than those obtained with other methods. (The relatively high yields from the dystrophic pectoralis at 0.12 and 0.25 yr are consistent with previously reported results (46,47)). The possibility exists that the variations in the extent of homogenization, caused by the increasing toughness of the dystrophic muscle, might have given rise to the observed variations in mitochondrial CPK activity.

Free energy for muscle function and maintenance is apparently entirely provided by the hydrolysis of ATP. If the mitochondrial CPK reaction is normally

an obligatory step in the overall process of oxidative ATP production in muscle (cf. Fig. 1), then the loss of this enzyme might be expected to cause a general deterioration of muscle function and structure, as in fact occurs in muscular dystrophy. However, it is likewise possible that the progressive loss of mitochondrial CPK activity reported here for dystrophic muscle is simply part of a degeneration triggered by other factors. Further experiments are necesaary to determine the direction(s) in which causality flows.

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