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Decreased rates of Ca^{2+}-dependent heat production in slow- and fast-twitch muscles from the dystrophic (*mdx*) mouse

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Abstract. Using a newly developed microcalorimetric approach to assess the rate of energy expenditure for intracellular [Ca^{2+}] homeostasis in isolated muscles at rest, we found this was lower in *mdx* than in control mouse muscles, by 62% and 29% in soleus and extensor digitorum longus, respectively. Differences in total and Ca^{2+}-dependent rates of specific heat production between *mdx* and control were enhanced during sustained, KCl-induced stimulation of energy dissipation. These results suggest that the low sacroplasmic energy status of dystrophic muscles is not due to any excessive energy expenditure for intracellular [Ca^{2+}] homeostasis.
Key words. Ca^{2+} transport; energy metabolism; skeletal muscle; sacroplasmic reticulum; Ca^{2+} channel; sacroplasmic Ca^{2+} homeostasis; muscular dystrophy.

A well-studied genetic disorder, which occurs in about 1/3500 human male newborns, and develops regularly in some animal models, prevents the production of a key plasmalemmal, cytoskeleton-related protein called dystrophin, which is normally found in detectable amounts in all muscular cells and some central neurons[1,2]. Whereas lack of dystrophin entails progressive muscular degeneration and death in early adulthood in human subjects (Duchenne muscular dystrophy) and dogs[3], in mice it only causes an early bout of muscle-fiber degeneration, followed by the regeneration of remarkably stable new cells[4], so that the lifespan of the *mdx* mouse is the same as that of the wild type[5]. We took advantage of the particular stability of adult *mdx*-mouse muscles to try to demonstrate the existence of a primary consequence of the lack of dystrophin on muscle-fibers' energy economy.

It is well known that the sacroplasmic energy status, as judged from chemical or spectroscopic determinations of high-energy phosphates, is subnormal in Duchenne muscular dystrophy[6] and its animal models[7–10]. This has been related implicitly, at least until quite recently[11], to the sacroplasmic free Ca^{2+} concentration (Ca^{2+}_i) being abnormally high[12], and as a consequence the energy demand for Ca^{2+}_i homeostasis being high too. This notion was reinforced by the finding that energy dissipation due to Ca^{2+} recirculation between sarcoplasmic reticulum (SR) and sacroplasm, which is by far the largest component of the energy cost of Ca^{2+}_i homeostasis[13,14], corresponds in normal cells to about 25% of metabolic rate and reaches about 45% under conditions of subthreshold-for-contracture stimulation of SR Ca^{2+} release[15]. Our purpose in the work reported here was to compare the rate of energy expended for Ca^{2+}_i homeosttasis in *mdx* and control muscles, using a recently tested rationale to evaluate this rate[15]. We found that it was lower in *mdx* than in control muscles, and that the ability of *mdx* muscles to increase their metabolic rate during moderate potassium-induced contractures was subnormal.

Methods

Control and *mdx* C57BL/10 mice[16] were taken from our own breeding colony (Dr G. Bulfield kindly provided us initially with 5 couples of homozygous *mdx* mice). The animals were kept at 24 °C ambient temperature and subjected to a daily 12-h light period, and they received unlimited water and food. After decapitation of an animal, both of its soleus and EDL muscles were dissected out intact with their tendons. They were placed on a stainless-steel frame, at physiological resting length, in the test chambers of twin microcalorimeters where they were superfused with Krebs-Ringer bicarbonate solutions for 5–8 h, starting with the 2- to 3-h period necessary for equilibration of the thermostatted calorimeters. All experiments were performed at 30 °C, so as to prevent anoxic core formation in the 5–15 mg superfused muscles. These were weighed at the end of the experiment, after removal of tendons and rapid blotting on filter paper; they were re-weighed after overnight drying at 70 °C and cooling down to ambient temperature.

The standard solution (mM: NaCl, 116.8; $NaHCO_3$, 25; KCl, 5.9; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 1.27; glucose, 5) was equilibrated at pH 7.4 with a 95% O_2–5% CO_2 gas mixture. So were the K^+-enriched solutions in which NaCl was decreased and partially replaced by sodium isethionate in order to keep constant both

the $[K^+][Cl^-]$ concentration product and osmolarity, and a Mg^{2+}-enriched solution in which 10 mM $MgCl_2$ replaced 15 mM NaCl (maintenance of osmolarity entailed a 15 mM decrease of sodium) and 5.5 mM sodium isethionate replaced 5 mM NaCl of the standard solution. The calmodulin inhibitor N-(6-aminohexyl) - 5 - chloro - 1 - naphthalenesulfonamide (W-7, Sigma, dissolved in methanol), the chemical phosphatase 2,3-butanedione monoxime (BDM, Sigma) or the sodium pump inhibitor ouabain (Fluka) were added to the solutions shortly before use.

The calorimetric signal, proportional to the total rate of muscle heat production, was an electrical potential difference between two series of six thermal gradient layers surrounding test and control chambers of the microcalorimeter, minus a blank difference recorded before introduction of the preparation into the test chamber. The proportionality constant between this signal and the preparation's heat production was determined for each channel, using a calibrated field-effect transistor as the heat source. At a chamber perfusion flow rate of 2 ml/min, this constant ranged from 10.2 mW/mV for the most sensitive channel to 10.7 mW/mV for the least sensitive one.

Skeletal muscles of adult *mdx* mice (with the exception of the diaphragm[17]), in contrast to those of DMD patients, exhibit only minimal infiltration[5,18–20]. Therefore heat production rates were standardized per unit wet weight of muscle and expressed in mW/g (means ± SEM, with the number of experiments in parentheses). Differences were analyzed using Student's t-test. For one set of data, the stepwise variable selection procedure of the Statgraphics statistical package (Statistical Graphics Corporation, 1985 edition) was used for the evaluation of the relative importance of muscle weight and mouse age on specific heat production rate.

Results

Basal rates of specific heat production. Mean values of specific heat production rates under basal conditions (Eb) in *mdx* and control soleus and EDL muscles are given in table 1 (first row). As explained in the methods section, normalization for wet muscle weight should not, in itself, introduce any significant bias in the comparison of the maintenance energy expenditure of *mdx* and control muscles. From recent data of Rooyackers and Smith[21], one can predict that normalization for muscle RNA or DNA contents, or for the fractional rate of protein synthesis, would enhance the observed difference in Eb between *mdx* and control soleus, and make an Eb difference appear between *mdx* and control EDL, as would normalization for the rate of glucose uptake by muscles[22] (and own measurements). On the other hand, normalization for non-collagen protein content[8] would slightly attenuate this difference. But the main factor which may affect the comparisons of Eb as presented in table 1 is the difference in fiber-type composition between *mdx* and control muscles. These differences apply mainly to fast-twitch muscles. Indeed, although there is some increase in the proportion of slow-twitch red (oxidative) fibers in *mdx* skeletal muscles with age, not seen in control muscles[23], histological and histochemical data reveal large, similar proportions of fast-twitch red (oxidative-glycolytic) fibers in adult *mdx* and control soleus muscles, as well as in *mdx* EDL muscles[24]. Thus, only the control mouse EDL, whose proportion of fast-twitch red fibers barely exceeds 40%, is typically composed of a majority of fast-twitch white (glycolytic) fibers. Only in this muscle did the dry/wet weight ratio correspond to that of a fast glycolytic muscle (table 2). The significantly larger proportion of fast-twitch red (oxidative-glycolytic) fibres in *mdx* than in control EDL[24] could therefore very well be responsible for the absence of Eb difference between *mdx* and control EDL. This notwithstanding, the known Eb difference between slow- and fast-twitch muscles[25], although smaller in *mdx* than in control mice, was statistically significant in both strains of mice.

Known differences in body and muscle growth rates between *mdx* and control mice made it difficult to exclude any influence of muscle size (unrelated to the effect of age) on basal, specific heat production. For example, at 160 days of age, *mdx* mice had a mean body weight of 35 g, about 15% larger than that of control mice, and the mean wet weight of one soleus muscle was close to 14 mg, 40% larger than that of the control muscle. From the results presented in figure 1 it can be calculated that at equal muscle weights of 10 mg, Eb of the *mdx* soleus was only about 30%

Table 1. Basal and Ca^{2+}-dependent rates of heat production in soleus and EDL muscles from *mdx* and control mice.

	Soleus		EDL		
	Control	*mdx*	Control		*mdx*
Basal E	4.45 ±0.09 (100)	2.92 ±0.06 (95)	2.51 ±0.06 (77)	NS	2.48 ±0.06 (64)
Effect of 10 mM BDM	−1.14 ±0.06 (23)	−0.43 ±0.02 (21)	−0.59 ±0.04 (16)		−0.42 ±0.03 (13)

Mean values ±SEM, in mW/g wet weight, with number of experiments in parentheses. Except the one marked NS, all differences between *mdx* and control are statistically significant ($p < 0.01$)

Table 2. Wet and dry weights of soleus and EDL muscles from *mdx* and control mice.

	Soleus			EDL	
	Control		*mdx*	Control	*mdx*
Wet weight	16.40 ±0.34 (115)		20.25 ±0.56 (112)	19.93 ±0.46 (85)	25.02 ±0.83 (66)
Dry weight	3.38 ±0.06 (115)		4.11 ±0.11 (112)	4.36 ±0.10 (85)	5.01 ±0.15 (66)
Dry/wet weight ratio	0.208 ±0.002 (115)	NS	0.205 ±0.002 (112)	0.220 ±0.003 (85)	0.202 ±0.002 (66)

Only control EDL muscles had a dry/wet weight ratio typical for normal white muscles, i.e., higher than that of normal red muscles by about 0.02[33]. Mean values ±SEM in mg, with number of measurements in parentheses. Except the one marked NS, all differences between *mdx* and control are statistically significant ($p < 0.001$).

lower than control, whereas the overall difference given in table 1 is about 35%. It should be kept in mind that 10-mg *mdx* soleus muscles were from younger mice than 10-mg control soleus muscles. Also, at least some if not all of the decrease of the specific rate of heat production with muscle weight was related to age. A stepwise variable selection procedure applied to the set of Eb values obtained from control soleus muscles (see upper regression in fig. 1), which took mouse age as well as muscle weight into account, revealed that the slope coefficient due to age was statistically significant, and that due to muscle weight was not.

Basal rates of Ca^{2+}-dependent heat production. An indirect estimate of the steady-state energy expenditure for sarcoplasmic Ca^{2+} *uptake* by SR[15] was obtained by measuring the decrease of E upon inhibition, brought about by 10 mM 2,3-butanedione monoxime (BDM), of Ca^{2+} *release* by SR (the recently evaluated effect of BDM on L-type calcium channels[26] is probably of little quantitative importance in skeletal muscle). This Ca^{2+}-dependent heat was lower in *mdx* than in control muscles of both types, in absolute terms as shown in table 1 (second row) as well as relative to Eb (about 15% and 17% of Eb in *mdx* soleus and EDL muscles, respectively, vs 26% and 24% in control muscles). This difference does not explain, in itself, the difference of Eb between *mdx* and control soleus muscles, to which it contributes only about 45%. This is illustrated in figure 2 which shows that, even under inhibition of SR Ca^{2+} release due to BDM, the specific heat production rate remains significantly lower in *mdx* than in control muscles. In another series of experiments, a Mg^{2+}-enriched superfusion solution was used as an alternative means to reversibly inhibit SR Ca^{2+} release[27]. Similar results were obtained: high Mg^{2+} levels inhibited E by

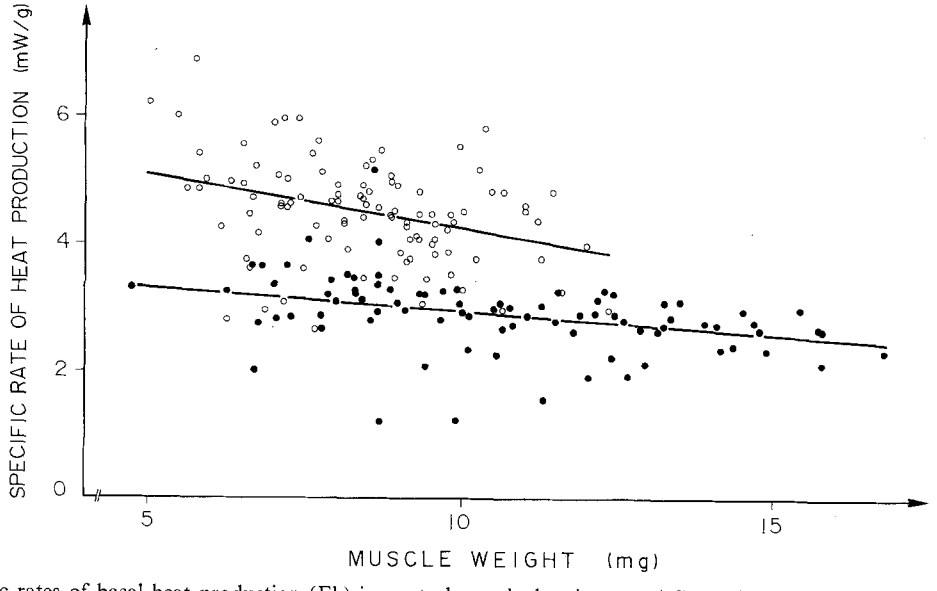

Figure 1. Specific rates of basal heat production (Eb) in control (○) and *mdx* (●) soleus muscles as functions of muscle weight. The slope difference between the two regressions (○: Eb = −0.084 wet wt + 5.91; ●: Eb = −0.038 wet wt + 3.7) was due to soleus muscle growth as a function of age being faster in *mdx* than in control C57BL/10 mice. The relatively large scatter of Eb values ($r = -0.34$ and -0.38, respectively) was partly inherent in the basal calorimetric signal having no other reference than a blank signal measured at the beginning of the experiment, before introduction of the preparation into the test chamber.

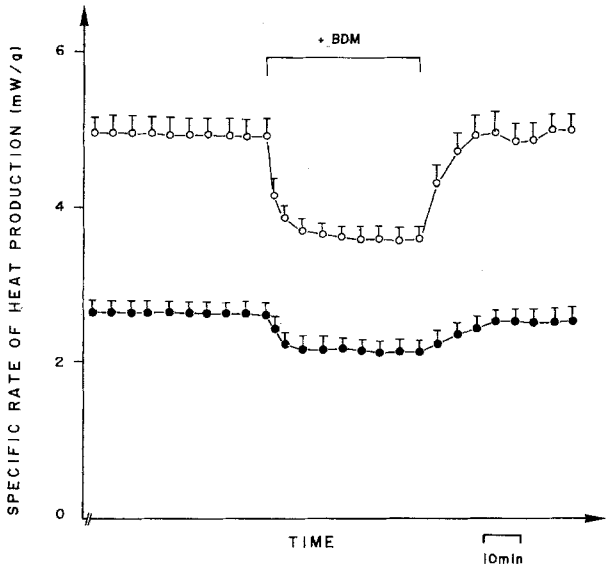

Figure 2. Time course of the effects of BDM (10 mM), used as an inhibitor of SR Ca^{2+} release, on specific heat production rates of *mdx* (●) and control (○) soleus muscles. These effects were sustained, and reversible upon removal of the drug from the superfusate. Mean results (with SEM indicated by vertical bars) of two series of seven experiments.

$11.3 \pm 0.6\%$ in *mdx* ($14.6 \pm 0.5\%$ with BDM) and by $22.9 \pm 2.1\%$ ($25.6 \pm 1.3\%$ with BDM) in control soleus muscles (10 comparisons). Again, under inhibition of SR Ca^{2+} release (here with high Mg^{2+}), there was a residual difference of E of about 1 mW/g between *mdx* and control muscles. This confirmed that not only energy expenditure devoted to $Ca^{2+}{}_i$ homeostasis was decreased in *mdx* muscles, but also the overall metabolic rate of the *mdx* soleus was decreased as compared to control.

Basal rates of energy dissipation by active Na^+, K^+ transport and Ca^{2+} transport across the sarcolemma. To confirm that in *mdx* and control C57BL/10 mice, as in other mouse strains, energy expenditure devoted to transsarcolemmal ion transport corresponds to but a small fraction of total metabolic rate, we measured the effects on E of the acute blocking of the Na^+, K^+ ATPase, or of the calmodulin-dependent Ca^{2+} ATPase. A prerequisite for both types of measurements was the prior inhibition of SR Ca^{2+} release. Indeed, the specific inhibitor of the Na^+, K^+ ATPase, ouabain, not only blocks active Na^+, K^+ transport but also increases SR Ca^{2+} release (and its energetic consequences) by a mechanism that is still unknown. On the other hand, calmodulin inhibition not only blocks active Ca^{2+} transport across the sarcolemma but also increases SR Ca^{2+} release by the removal of the calmodulin-dependent control of this process[28]. We therefore measured the effects of the inhibitors (1 mM of ouabain or 30 μM of the calmodulin inhibitor W-7) in presence of 10 mM BDM so as to prevent the secondary increase of Ca^{2+}-dependent heat production. As expected, the immediate and sustained decrease of Eb under the effect of either plasma-membrane ATPase inhibitor was small: 7–9% of Eb for ouabain (means of 6–8 experiments in each muscle type of both *mdx* and control mice), and 1–3% of Eb for W-7 (means of 4–7 experiments in each muscle type of both *mdx* and control mice). There was a small but statistically significant difference between the acute ouabain effects on *mdx* and control soleus muscles' heat production rates (-0.24 ± 0.02 and -0.44 ± 0.06 mW/g, respectively, $p < 0.01$).

Subthreshold-for-contracture stimulation of Ca^{2+}-dependent heat. Under stimulation through an increase of extracellular K^+ [15,29] to 11.8 mM the sustained, specific rate increase of heat production was entirely Ca^{2+}-dependent, so that *total* Ca^{2+}-dependent heat, taken as the effect of 10 mM BDM under 11.8 mM extracellular K^+ ($K^+{}_0$), became a significantly larger fraction of E than under basal conditions. But the differences of Ca^{2+}-dependent heat between *mdx* and control muscles persisted, in absolute terms as shown in table 3 (second

Table 3. Increases of heat production rate induced by elevations of extracellular K^+ (K_0^+) to 11.8 and 20.9 mM (by adding 5.9 and 15 mM K_0^+, respectively), and Ca^{2+}-dependent heat (effect of 10 mM BDM) under these conditions, in soleus and EDL muscles from *mdx* and control mice.

	Soleus Control		*mdx*	EDL Control	*mdx*
11.8 mM $K^+{}_0$	0.65 ±0.05 (23)	NS	0.52 ±0.03 (18)	0.60 ±0.05 (19)	0.32 ±0.04 (13)
Effect of 10 mM BDM under 11.8 mM $K^+{}_0$	−1.89 ±0.12 (11)		−0.96 ±0.05 (10)	−1.06 ±0.09 (8)	−0.64 ±0.04 (7)
20.9 mM $K^+{}_0$	5.06 ±0.56 (7)		3.24 ±0.25 (7)	5.64 ±0.37 (4)	1.82 ±0.19 (4)
Effect of 10 mM BDM under 20.9 mM $K^+{}_0$	−4.56 ±0.33 (6)		−3.14 ±0.24 (6)	−5.26 ±0.37 (4)	−1.84 ±0.18 (4)

Mean values ±SEM, in mW/g wet weight with number of experiments in parentheses. Except the one marked NS all differences between *mdx* and control are statistically significant ($p \leq 0.01$)

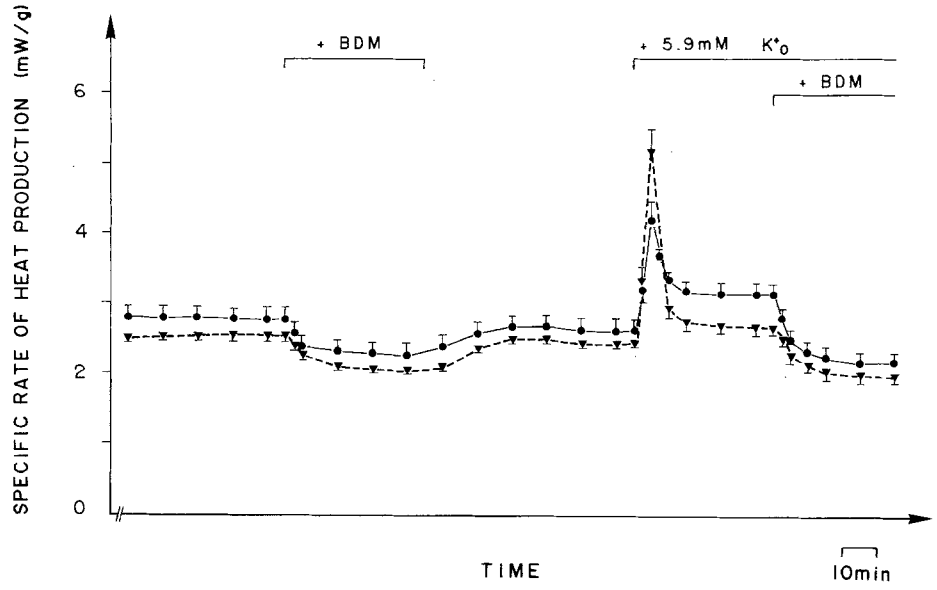

Figure 3. Time course of the effects of BDM on the specific rate of heat production (E), before and during a subthreshold-for-contracture increase of extracellular potassium to 11.8 mM K^+_0 (by the addition of 5.9 mM K^+_0 at constant osmolarity and $[K^+][Cl^-]$ product), in soleus (●) and EDL (▼) muscles from *mdx* mice. The thermogenic response to potassium comprised an initial burst of E (larger for the fast-twitch EDL than for the slow-twitch soleus) followed by a sustained phase during which excess heat production, with respect to Eb, was about twice as large for soleus as for EDL muscles. For both types of muscle, excess E during the sustained phase was entirely Ca^{2+}-dependent (i.e., BDM suppressible). Mean results (with SEM indicated by vertical bars) of two series of seven experiments.

row) as well as relative to total E (about 28% and 23% in *mdx* soleus and EDL muscles, respectively, vs 37% and 34% in the corresponding control muscles). Under these conditions, the difference in E between *mdx* and control was statistically significant for EDL as well as for soleus muscles. Also, as shown in figure 3, the sustained increase of Ca^{2+}-dependent heat was relatively smaller in EDL than in soleus muscles from *mdx* mice (53% vs 126%).

Total and Ca^{2+}-dependent rates of heat production under moderate potassium-induced contracture. Under the much stronger stimulation of muscle energy metabolism induced by an increase of K^+_0 to 20.9 mM (compare row 3 to first row of table 3), the differences in total and Ca^{2+}-dependent rates of heat production between *mdx* and control muscles of both types became evident. This is also true for the differences, between *mdx* EDL and soleus muscles, which already appeared under 11.8 mM K^+_0. Figure 4 shows the large difference of total, specific rate of heat production between *mdx* and control EDL muscles.

Discussion

Measurements of basal heat production rate (Eb) revealed that the energy cost of maintenance is lower than normal in all fiber types of the *mdx* mouse. Indeed, the lack of any Eb difference between *mdx* and control EDL muscles can be taken to result simply from the former being significantly enriched with fast-twitch red fibers (large oxidative capacity[30]) and, correspondingly, impoverished in fast-twitch white ones (low basal metabolic and protein[31] turnover rates) as compared to control EDL (see also ref. 32). More striking was the evidence that the Ca^{2+}-dependent part of E is significantly smaller in *mdx* than in control muscles of both types, under basal as well as subthreshold-for-contracture conditions. This Ca^{2+}-dependent heat was also a smaller proportion of E in *mdx* than in control muscles, which suggests that this component of the metabolic rate is particularly sensitive to any shortage of energy. The same was observed under moderate potassium-induced contracture, where the difference between *mdx* and control was more marked in the fast-twitch than in the slow-twitch muscle, as regards both the overall thermogenic response to potassium and the Ca^{2+}-dependent part of E. This last finding indicates that despite the first impression conveyed by the results obtained under basal conditions, slow-twitch *mdx* muscles appear to have a greater ability than fast-twitch ones to increase their total and Ca^{2+}-dependent rates of specific heat production. That fast-twitch do not keep up with slow-twitch muscles may be related to the observation that, in contrast to the soleus, the EDL of the adult *mdx* mouse shows reduced maximum velocity of shortening, as well as reduced isometric twitch and tetanic tensions, with respect to control EDL[24].

The present results indicate that the abnormally large cellular calcium *content* of dystrophic muscle fibers (essentially protein-bound calcium in the SR matrix) is not accompanied by any large intensity of the intracellular

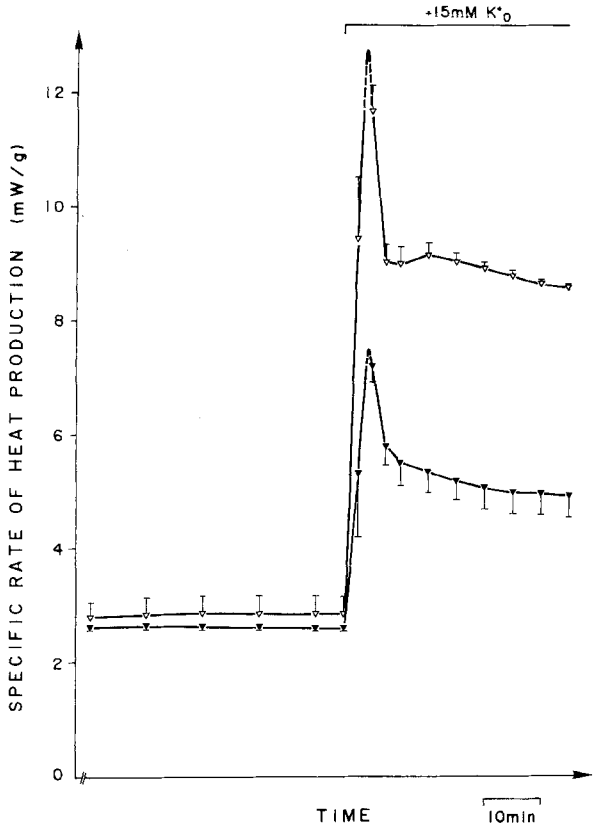

Figure 4. Time course of the specific heat production rates of *mdx* (▼) and control (▽) EDL muscles before and during a moderate potassium-induced contracture (elevation of K^+_o to 20.9 mM by the addition of 15 mM K^+_o at constant osmolarity and extracellular $[K^+][Cl^-]$ product). Mean results (with SEM indicated by vertical bars) of two series of four experiments, showing that the sustained thermogenic response to potassium was markedly reduced in *mdx* as compared to control muscles. Contrary to what was observed during subthreshold-for-contracture stimulations (fig. 3), not all of the thermogenic response to potassium was BDM suppressible, as can be seen from the total effect of BDM under 20.9 mM K^+_o not exceeding this response (compare last two rows with first two rows of table 3).

Ca^{2+} *movement*, which in fact is reduced with respect to normal. Since no intrinsic deficiency of the sarcoplasmic Ca^{2+} clearance processes has ever been found in either DMD patients or *mdx* mice[12,32], the simplest interpretation of the present microcalorimetric data is that SR matrix repletion with calcium in the absence of muscle-cell excitation, and the concomitant limitation of SR Ca^{2+} uptake and energy expenditure, is the consequence of a decreased SR Ca^{2+} release. No abnormality of the dihydropyridine receptor function or any other component of the excitation-contraction coupling process has been described in dystrophic muscle. Therefore one may speculate that the conductance of the adenine nucleotide-activated Ca^{2+} channels of the SR membrane, which is very dependent on ATP concentration on the sacroplasmic side[27], is functionally limited in situ by the low sarcoplasmic energy status. This subnormal energy status of dystrophic fibers would, in turn, result from some primary defect of energy production as first suggested by the present data. That *mdx* mice have a qualitatively normal muscle function is entirely compatible with this view. Indeed, their SR Ca^{2+}-*re*uptake processes *do* respond (however subnormally from a quantitative point of view) to stimulations of SR Ca^{2+} release, because immediately after some of their SR Ca^{2+} overload has been released rapid Ca^{2+} reuptake can occur[12].

To conclude, the present quantitative analysis revealed the existence of some defect of energy metabolism in dystrophic muscle cells, and the simplest interpretation of the results points to this defect being a primary consequence of the lack of dystrophin. This may cast new light on the function of this protein.

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