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COMPARTMENTATION OF HIGH-ENERGY PHOSPHATES IN RESTING AND WORKING RAT SKELETAL MUSCLE

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The subcellular distribution of high-energy phosphates in various types of skeletal muscle of the rat was analysed by subfractionation of tissues in non-aqueous solvents. Different glycolytic and oxidative capacities were calculated from the ratio of phosphoglycerate kinase and citrate synthase activities, ranging from 25 in m. soleus to 130 in m. tensor fasciae latae. In the resting state, the subcellular contents of ATP, creatine phosphate and creatine were similar in m. soleus, m. vastus intermedius, m. gastrocnemius and m. tensor fasciae latae but, significantly, a higher extramitochondrial ADP-content was found in m. soleus. A similar observation was made in isometrically and isotonically working m. gastrocnemius. The extramitochondrial, bound ADP accounted fully for actin-binding sites in resting fast-twitch muscles, but an excess of bound ADP was found in m. soleus and working m. gastrocnemius. The amount of non-actin-bound ADP reached maximal values of approx. 1.2 nmol/mg total protein. It could not be enhanced further by prolonged isotonic stimulation or by increased isometric force development. It is suggested that non-actin-bound ADP is accounted for by actomyosin-ADP complexes generated during the contraction cycle. Binding of extramitochondrial ADP to actomyosin complexes in working muscles thus acts as a buffer for cytosolic ADP in addition to the creatine system, maintaining a high cytosolic phosphorylation potential also at increasing rates of ATP hydrolysis during muscle contraction.

Introduction

It is well known that skeletal muscle is composed of different fiber types, 'slow-twitch-oxidative' fibers with high oxidative and low glycolytic capacity, 'fast-twitch-oxidative-glycolytic' fibers with moderately high oxidative as well as glycolytic capacities, and 'fast-twitch-glycolytic' fibers with high glycolytic and low oxidative capacities [1–3]. Such differences can be expressed as relations of the enzymatic capacity of glycolysis to that of critic acid cycle, so-called 'system relations' [4].

One problem investigated in the present work is whether in view of the substantial variation in metabolic capacities in the different types of muscle there also exist differences in subcellular high-energy phosphate composition. Therefore, extramitochondrial and mitochondrial high-energy phosphate contents were determined in rat skeletal muscles of distinct fiber composition using non-aqueous fractionation of freeze-clamped and freeze-dried tissue [5].

The determination of extramitochondrial adenine nucleotide contents is complicated by the fact that a fraction of ATP as well as a considerable amount of extramitochondrial ADP is bound to contractile proteins in skeletal muscle

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[6,7]. Binding of ADP to cytosolic proteins has also been reported for liver [8]. Whereas contradicting results exist regarding the binding of ATP or ADP to myosin in the resting muscle [6,9,10] it is generally accepted that ADP is tightly bound to actin in muscle under all physiological conditions [7,11]. Based on current contraction models, it can further be assumed that during muscle work further ADP is bound to actomyosin complexes [12,13]. We therefore investigated whether bound ADP is found in working muscles which cannot be assigned to actin binding sites. Extramitochondrial non-actin-bound ADP was calculated from the total ADP measured by subtracting mitochondrial ADP determined with the non-aqueous fractionation method [5], actin-bound ADP taken from [14] and free extramitochondrial ADP calculated from the creatine kinase equilibrium. The data indicate that non-actin-bound extramitochondrial ADP rises on increasing muscle work up to approx. 1.2 nmol/mg total protein.

Methods

Male rats of the Wistar-strain (body weight 180–280 g), fed with a standard diet (Altromin, Lage, F.R.G.) and water ad libitum were used. They were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The tissue surrounding the muscles was removed carefully to avoid damage of the blood vessels and the muscles were freeze-clamped in situ with aluminium clamps precooled in liquid nitrogen [15]. The frozen tissue was 1–1.5 mm thick and was pulverized in liquid nitrogen and freeze-dried at −40°C.

The freeze-dried muscle tissue was fractionated utilizing density gradient centrifugation in non-aqueous media according to Ref. 5 with the following modifications. About 0.2 g of freeze-dried tissue was sonified for 4 min every 5 s at 5-s intervals (150 W; Branson B12 instrument) in a heptane/carbon tetrachloride medium ($d = 1.23$ kg/l). The muscle suspension was cooled continuously in a mixture of heptane and solid CO_2.

The homogenate was then filtered through a column (10 × 2 cm) containing glass beads (diameter 0.45–0.5 mm), which retained connective tissue. However, as some cytosolic protein also tended to be retained on the column, for determination of protein distribution the filtration step was omitted. The subcellular distribution of the metabolites was found to be similar with and without the glass-bead column, as tested with m. soleus ($n = 6$). This finding is to be expected, since metabolites and proteins of the subspaces are not separated during fractionation (see calculation) in non-polar media and since metabolites of each compartment are referred to their compartmental protein, respectively. Therefore, data obtained with and without the column treatment were pooled.

The homogenate was centrifuged for 5 min at 19500 × g and all but 1–2 ml of the supernatant was decanted. The pellet was resuspended in the remaining supernatant, and then layered on top of a logarithmic density gradient (heptane/carbon tetrachloride, $d = 1.308$–1.334 kg/l) and centrifuged for 3 h at 19500 × g. The gradient yielded eight fractions, each containing different proportions of extramitochondrial and mitochondrial protein. Each fraction was divided into two aliquots. From one aliquot the activities of the extramitochondrial marker enzyme phosphoglycerate kinase, and of the mitochondrial marker enzyme citrate synthase as well as the protein content were determined as described in Ref. 16. In the second aliquot, the contents of ATP, ADP, creatine phosphate and creatine were measured using enzymatic analyses [16,17].

Calculation

The determination of extramitochondrial and mitochondrial metabolite contents and of protein distribution is based on the determination of metabolite contents and protein contents in fractions from the density gradient which contain extramitochondrial as well as mitochondrial protein in different proportions. The relative proportion of extramitochondrial and mitochondrial compartments in each fraction can be estimated from the activities of marker enzymes, i.e., phosphoglycerate kinase (extramitochondrial space) and citrate synthase (mitochondrial matrix). The means that those cellular components and their respective metabolites contribute to the extramitochondrial space, which are surrounded by the soluble space of phosphoglycerate kinase; this includes the cellular membrane, myofibrillar space, sarcoplasmic re-

ticulum, nuclear space, etc. The mitochondrial space consists of the matrix space and of the inner mitochondrial membrane which are 'labelled' by citrate synthase. This is based on the assumption that soluble enzymes and metabolites in each compartment are not separated from their respective compartment during fractionation of the tissue in solvents of low polarity.

From the specific activities of phosphoglycerate kinase and citrate synthase and the metabolite content in each fraction, it is extrapolated to metabolite contents in mitochondria and extramitochondrial space according to Ref. 5. Data are expressed per mg compartmental protein. The subcellular distribution of protein is calculated principally in the same way; however, only the relative distribution is obtained (in percent) since subcellular water spaces cannot be determined by this method. Mitochondrial protein was determined to be $18 \pm 5\%$ ($n = 12$) of cellular protein in m. soleus and in m. gastrocnemius, and this value was regarded to be representative for all muscle types studied. On the basis of the protein distribution obtained by fractionation and using a value of 4.3 for the relation wet weight/protein obtained for m. gastrocnemius (six determinations) the data in Table II were referred to total protein. The content of actin binding sites of 0.5 μmol/g wet weight from Ref. 14 was also referred to mg total protein on the same basis and calculated to be 2.2 nmol/mg total protein.

Cellular pH measurement

The extramitochondrial pH in resting m. gastrocnemius was determined by means of the subcellular distribution of [^{14}C]DMO (5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione) [18] in freeze-clamped muscles from perfused rat hindquarter [19] or anaesthetized rats in vivo. [^{14}C]DMO, a non-metabolizable weak acid, was either added to the perfusion medium (40 μCi/10 ml per min) 10 min before freeze-clamp or injected into the tail vein of anaesthetized rats (300 μCi/kg rat) 30 min before freeze-clamping the muscle in situ. The same value within experimental error of 7.0 ± 0.1 was obtained with both methods and therefore the experiments were averaged ($n = 5$). The value is in fair agreement with recent data obtained by NMR as well as microelectrode measurements [20,21]. A pH of 7.0 was also used for muscles isometrically working for 6 s. This assumption seems to be reasonable, since no pH changes were detected in skeletal muscles, within the first minute of stimulation, by ^{31}P-NMR (Shoubridge, E. and Radda, G.K., personal communication). If the duration of stimulation exceeded 1 min, an extramitochondrial pH of 6.6 was used as determined by ^{31}P-NMR (same reference).

Stimulation of m. gastrocnemius

In each experiment, the m. gastrocnemius of the left leg was stimulated while the contralateral one was taken as control.

In isotonic experiments, muscles were stimulated electrically via n. ischiadicus with a frequency of 5 Hz, pulse width 1 ms and a variable amplitude of 1–3 V. The muscles were freeze-clamped after 0.5, 1.0, 3.0 and 5.0 min.

In isometric experiments, muscles were stimulated via n. ischiadicus by square waves with a frequence of 100 Hz, pulse width of 1 ms and variable amplitude. The muscles were freeze-clamped in situ after 6 s of stimulation. The force generated in isometric experiments was measured with a Harvard isometric transducer.

Materials

Creatine kinase was obtained from Sigma Chemical, St. Louis, MO; other enzymes and coenzymes were purchased from Boehringer, Mannheim, F.R.G.; sodium pentobarbital (Nembutal) was from Abbott, Neuilly-sur-Seine, France. Radiochemicals were from Amersham Buchler, Braunschweig, F.R.G. All other chemicals were from E. Merck, Darmstadt, F.R.G. and were of the highest purity available.

Results

'System relations' of glycolysis and citric acid cycle in skeletal muscle

The system relation between glycolysis and citric acid cycle was calculated from the ratio of the activities of phosphoglycerate kinase and citrate synthase, respectively (Table I). The values correlate well with the functions of the different types of skeletal muscle. It is highest in m. tensor fasciae

TABLE I

SYSTEM RELATION BETWEEN GLYCOLYSIS AND CITRIC ACID CYCLE AND FIBER POPULATION IN DIFFERENT RAT SKELETAL MUSCLES

The system relation between glycolysis and citric acid cycle represents the ratio of activities of phosphoglycerate kinase and citrate synthase (±S.D.). Fiber populations (in percent) are shown for comparison; data are from Ref. 3.

	M. soleus	M. vastus intermed.	M. gastrocnemius	M. tensor fasc. lat.
Phosphoglycerate kinase citrate synthase	25±4	32±4	80±14	130±15
Fiber populations				
slow-twitch-oxidative	84	36	5	0
fast-twitch-oxidative-glycolytic	16	64	37	6
fast-twitch-glycolytic	0	0	58	94

latae, being 130, and lowest in m. soleus, being 25. M. vastus intermedius and m. gastrocnemius lateralis are intermediate between these two extremes with regard to fiber composition [3] as well as to system relation. The enzymatic capacities determined are confirmed by in vivo measurements of flux rates. For example, flux through the enolase reaction was measured to be about 9-times

TABLE II

TOTAL AND SUBCELLULAR METABOLITE CONTENTS IN SKELETAL MUSCLES OF THE RAT

Values are expressed as nmol/mg protein and are the average of 9–17 determinations±S.D. from 2–3 pools, each containing the tissue of ten muscles of a given type. The mean values of each pool differed not significantly for any metabolite. CrP, creatine phosphate; Cr, creatine; *P* (vs. m. soleus; Student's *t*-test): [a] $P < 0.0005$; [b] $P < 0.005$; [c] $P < 0.025$.

	M. soleus slow-twitch oxidative	M. vastus intermedius fast-twitch oxidative glycolytic	M. gastrocnemius fast-twitch oxidative glyc.	M. tensor f.l. fast-twitch glycolytic
Total				
Creatine-phosphate	69 ± 9	90 ± 8	93 ±12	87 ± 9
Creatine	50 ± 9	73 ±12	61 ± 9	74 ±14
CrP/Cr	1.4± 0.2	1.2± 0.2	1.5± 0.3	1.2± 0.1
ATP	29 ± 3	39 ± 4	37 ± 5	42 ± 5
ADP	5.3± 1.2	4.6± 1.6	4.6± 0.2	4.6± 0.7
ATP/ADP	5.5± 1.0	8.5± 1.6	8.0± 1.0	9.1± 1.8
Mitochondrial				
Creatine-P	< 3	< 3	< 3	< 3
Creatine	39 ±10	68±11	76 ±16	91±23
CrP/Cr	–	–	–	–
ATP	23 ± 5	23 ± 4	31 ± 6	25 ± 4
ADP	9.3± 3.3	9.5± 1.7	11 ± 2	13 ± 4
ATP/ADP	2.5± 0.7	2.4± 0.5	2.8± 0.8	1.9± 0.6
Extramitochondrial space				
Creatine-P	89 ±13	110 ±10	115 ±18	111 ±18
Creatine	58 ± 9	74 ±12	60 ± 9	77 ±17
CrP/Cr	1.5± 0.3	1.5± 0.2	1.9± 0.3	1.4± 0.3
ATP	33 ± 2	42 ± 3 [a]	41 ± 7 [a]	46 ± 6 [a]
ADP	4.4± 1.4	3.1± 1.0 [b]	3.1± 0.5 [b]	3.3± 0.9 [c]
ATP/ADP	7.5± 2.1	13.6± 2.3	13.2± 2.6	13.8± 3.9

higher in white than in red muscles of the rabbit [22].

Subcellular phosphagen contents in resting muscles

Contrary to the distinct system relations in the different skeletal muscles, the contents of ATP, creatine phosphate and creatine are generally similar in all muscle types studied (Table II). In soleus muscle the total content of each of ATP, creatine phosphate and creatine is slightly lower and that of ADP is higher than in the other muscles. The mitochondrial phosphagen content is similar in all muscles studied; however, creatine increases from m. soleus to m. tensor fasciae latae. In confirmation of earlier measurements in isolated fibroblastoid rat-heart cells [17] and isolated working guinea-pig heart [23], but in contrast to measurements in rat heart [24], no creatine phosphate was found in mitochondria. It seems reasonable, however, that creatine phosphate, being a polar molecule, is excluded from mitochondria, since no translocator for it is known in the mitochondrial membrane, and creatine kinase is likewise excluded from the mitochondrial matrix [25]. In the extramitochondrial space, a similar content of creatine phosphate and creatine was found in all muscle types. In m. soleus, the extramitochondrial content of ATP is significantly lower and that of ADP is significantly higher than in the other muscles. As a consequence, the extramitochondrial ATP/ADP ratio of contents is distinctly lower in soleus muscle than in the others (Table II). However, in contrast to the low ATP/ADP ratio of contents, the extramitochondrial CrP/Cr ratio in the slow-twitch muscle is similar to that found in the other muscle types.

Compartmentation of ADP

(a) Mitochondrial ADP. The mitochondrial contents of ADP were determined in four different skeletal muscles ranging from 1.7 ± 0.6 nmol/mg total protein to 2.3 ± 0.7 nmol/mg total protein (Table III). Experiments on isotonically as well as isometrically working m. gastrocnemius also showed similar mitochondrial ADP contents of 2.2 ± 0.7 and 1.8 ± 0.1 nmol/mg total protein, respectively.

(b) Extramitochondrial ADP. In resting muscles

TABLE III

SUBCELLULAR DISTRIBUTION OF ADP IN DIFFERENT SKELETAL MUSCLES AND UNDER DIFFERENT FUNCTIONAL STATES (Data are given as nmol/mg total protein; means ± S.D.)

Data for mitochondrial and extramitochondrial ADP in resting muscles are from Table II. Values for extramitochondrial non-actin-bound ADP were obtained by subtracting actin-bound ADP (2.2 nmol/mg total protein, see Methods) and free extramitochondrial ADP from total extramitochondrial ADP. Values for free extramitochondrial ADP content are calculated from the mass action ratio of the creatine kinase reaction using extramitochondrial contents from Table II for resting muscles (for working muscles data not shown):

$$K_{CK} = \frac{[\sum ATP] \cdot [\sum Cr]}{[\sum ADP] \cdot [\sum CrP] \times [H^+]}$$

$K_{CK} = 1.66 \times 10^9\ M^{-1}$, $T = 38°C$, $I = 0.25$, $[Mg^{2+}]_{free} = 10^{-3}$ M [33]; pH = 7.0 and 6.6 for resting and working state, respectively.

	M. soleus resting state	M. vastus resting state	M. gastrocnemius			M. tensor f.l. resting state
			resting state	isometric state (5 min)	isotonic state (0.5–5 min)	
Mitochondrial	1.7 ±0.6	1.7 ±0.3	2.0 ±0.4	1.8 ±0.1	2.2± 0.7	2.3 ±0.7
Extramitochondrial						
Total	3.6 ±1.1	2.5 ±0.8	2.5 ±0.4	3.6 ±0.8	3.9 ±0.8	2.7 ±0.7
Free	0.10±0.03	0.18±0.03	0.12±0.02	0.20±0.04	0.59±0.23	0.16±0.05
Non-actin-bound	1.3 ±1.1	0.1 ±0.8	0.2 ±0.4	1.2 ±0.8 [a]	1.1 ±0.8 [a]	0.3 ±0.7

[a] $P < 0.005$ versus resting state, Student's t-test.

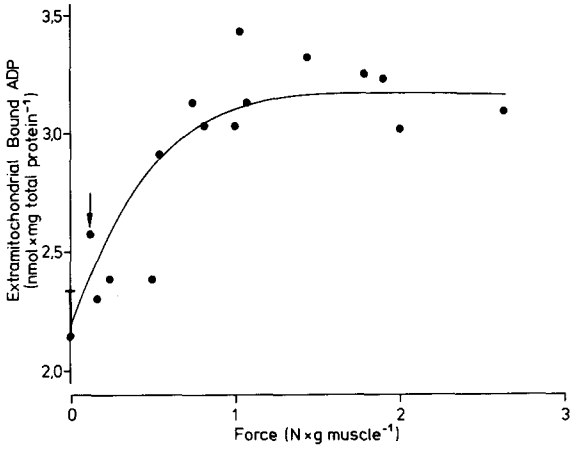

Fig. 1. Relationship between extramitochondrial, bound ADP and isometric force development. Extramitochondrial, bound ADP was calculated by subtracting the mitochondrial and free extramitochondrial ADP content from the total cellular ADP content. Each point represents the mean of 3–5 determinations from one animal. The control value is the average from the controls of all 14 experiments (mean ± S.D.). The force values on the abscissa are given as N/g muscle developed via the Achilles tendon at the moment of freeze-clamp. The arrow refers to an experiment discussed on page 123.

containing predominantly fast-twitch fibers, all bound extramitochondrial ADP can be assigned to actin-binding sites within experimental error (Table III). Contrary to this, considerable amounts of non-actin-bound ADP are obtained for m. soleus and working m. gastrocnemius. The amount of non-actin-bound extramitochondrial ADP makes up to 30% of total extramitochondrial ADP in both cases, i.e., around 1.2 nmol/mg total protein.

The free cytosolic ADP content as calculated from the creatine kinase equilibrium is lowest in the resting state and increases to the highest value during isotonic contraction (Table III).

(c) Relationship between non-actin-bound ADP and force development in isometrically stimulated m. gastrocnemius. M. gastrocnemius were stimulated for 6 s, causing different levels of isometric force development. Since we observed no significant changes in mitochondrial ADP content in m. gastrocnemius under resting, isotonic and isometric working conditions the muscles for this experiment were not fractionated, but the extramitochondrial ADP content was calculated by subtracting the mean value of the mitochondrial ADP contents (2.0 nmol/mg total protein) from the measured total contents. Increasing amounts of non-actin-bound ADP with increasing force development were observed (Fig. 1). The value for non-actin-bound ADP increased up to 1 nmol/mg total protein; no further increase was observed when the force increased.

Discussion

The application of the technique of fractionation of tissue in non-aqueous solvents provided a suitable method to study the changes of free and bound ADP in muscle under different physiological conditions. With this method, metabolites in the mitochondrial and extramitochondrial space can be separated in intact tissue without disturbing the metabolic state fixed by freeze-clamping, while there is no possibility of discerning between myofibrillar, sarcoplasmic reticulum or cytosolic space. In contrast, non-invasive NMR measurements in vivo can detect free metabolites of the soluble extramitochondrial space, i.e., the cytosol, but not metabolites of the mitochondrial space or those bound to proteins. Thus, in the particular case of studying compartmentation of ADP in muscle, it is possible with the method employed here to discriminate mitochondrial (free + bound) ADP, extramitochondrial free ADP calculated from extramitochondrial creatine phosphate/creatine ratios, extramitochondrial actin-bound and non-actin-bound ADP.

Binding of ATP and ADP in the resting muscle

Based on kinetic studies with isolated myosin, heavy meromyosin or myosin subfragment 1 [9,10,26], it was concluded that in the steady state in resting muscle, myosin exists predominantly as myosin-ADP complex. Ferenczi [9] found that 95% of myosin-S1 heads may contain bound ADP. He confirmed his notion by comparing the amount of cellular ADP with that of actin- and myosin-binding sites in frog muscles. The sum of binding sites agreed fairly well with the content of ADP. However, subtraction of cytosolic free ADP and correction for mitochondrial ADP (accounting for about 40% of total, Table III) were omitted in Ref. 9. If

these ADP pools are taken into account, it is seen that 80–90% of extramitochondrial ADP is bound to actin in resting fast-twitch muscles (Table III, m. vastus; m. tensor; m. gastrocnemius). Thus, similar to findings of Bárány and Bárány [6] in frog muscle, our data indicate that only a minor part of myosin can exist as myosin-ADP complex in resting rat skeletal muscles. Bárány and Bárány further found that ATP inhibited competitively *N*-ethyl[^{14}C]maleimide incorporation into myosin nucleotide binding sites in resting muscle and concluded from this that myosin binds ATP under this condition.

Thus, direct measurements and binding studies in intact muscle come to conclusions which differ from kinetic studies of myosin in solution [9,10,26]. This controversy may be explained on the basis of thermodynamic considerations by Eisenberg and Hill [12], saying that conformational changes occurring upon nucleotide association or dissociation from myosin in solution, tend quickly towards the state of minimal energy, i.e., myosin-products complex, compared to myosin in intact muscle, since there is less steric impairment in solution.

Binding of extramitochondrial ADP in working muscles

At the transition to the working state in m. gastrocnemius, an additional pool of extramitochondrial, bound ADP is formed up in isotonically as well as isometrically working muscle (Table III). In isotonically working muscle, non-actin-bound ADP (approx. 1.1 nmol/mg total protein) seems to be independent of the duration of work (0.5–5.0 min).

In isometrically working m. gastrocnemius non-actin-bound ADP increased from about zero in the resting state to about 1 nmol/mg total protein at a force development of 1N/g muscle (Fig. 1). Further increase in force does not raise non-actin-bound ADP. Moreover, the content is determined only by the force level at the time of freeze-fixation, since its value is independent of the course of force development during the 6 s stimulation phase. For example, an experiment where the muscle developed a rapid increase in force up to 1.1 N/g followed by a decline to 0.14 N/g at the time of freeze-fixation, showed an amount of bound extramitochondrial ADP which fits to the corresponding force level of 0.14 N/g (Fig. 1, arrow). Thus, in the region where non-actin-binding sites are not saturated with nucleotides, a distinct relation exists between muscle work and non-actin-bound ADP.

Nature of non-actin-bound ADP

The saturation value for non-actin-bound ADP of approx. 1.2 nmol/mg total protein is close to the values reported for myosin nucleotide binding sites of 1.0–1.8 nmol/mg total protein in the muscle cell [6,27,28]. According to current models of muscle contraction [12,13], the dissociation of the hydrolysis products (i.e., ADP and phosphate) is the rate-limiting step in the contraction cycle leading to an accumulation of actomyosin-ADP- or actomyosin-ADP-phosphate complexes. One of these complexes is considered to be the force-producing state [12]. Since the amount of actomyosin complexes cannot exceed that of myosin subfragment-1 heads, it has to be in the range of myosin nucleotide binding sites. Therefore, on the basis of the finding that the amount of non-actin-bound ADP increases up to a constant value of about 1.2 nmol/mg total protein in isometric and isotonic stimulation experiments (Table III, Fig. 1), it is reasonable to assume that the non-actin-bound ADP may be assigned to actomyosin-ADP complexes.

Contrary to the findings in muscles containing predominantly fast-twitch fibers, m. soleus contains non-actin-bound extramitochondrial ADP even in the resting state (Table III). M. soleus as a tonic muscle seems to be active, even in the resting state, as shown by measurements of energy-rich phosphates [29] and by electromyography [30] as well as force recordings [31] in the cat, and therefore a considerable amount of actomyosin-ADP complexes may be present, even at rest.

The increase in the amount of bound ADP in the cytosolic space during enhanced ADP production, i.e., muscle work, may be viewed as a first line of defence against perturbation of the cytosolic phosphorylation potential in the view of damping, whereas drawing on the creatine system is a second line of defence. Thus, in addition to the free thermodynamic concentrations it is important to identify the free and bound proportions of a metabolite. As has been shown previously for the

buffering of cytosolic NADH concentration in liver by binding to suitable sites [32], the buffering of cytosolic ADP by extra binding sites in muscle as detected here is a useful device for maintenance of cellular homeostasis in energy metabolism despite different functions of muscles (e.g., m. soleus vs. m. tensor). A special feature here is that the capacity of available binding sites is expanded upon an increase in functional demand, somewhat in analogy to the functional adaptation in capacity of buffering the hydrogen ion by hemoglobin in response to oxygenation-deoxygenation, known as the Bohr effect.

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