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Review

Functional coupling as a basic mechanism of feedback regulation of cardiac energy metabolism

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The more perfect the organization, the more the functions are distinct and determined, and the more they are dependent on one another

Hegel, G.W.F. (1907)
Vorlesungen über die philosophie der Geschichte.
F. Brunstad, Leipzig

Abstract

In this review we analyze the concepts and the experimental data on the mechanisms of the regulation of energy metabolism in muscle cells. Muscular energetics is based on the force–length relationship, which in the whole heart is expressed as a Frank–Starling law, by which the alterations of left ventricle diastolic volume change linearly both the cardiac work and oxygen consumption. The second basic characteristics of the heart is the metabolic stability – almost constant levels of high energy phosphates, ATP and phosphocreatine, which are practically independent of the workload and the rate of oxygen consumption, in contrast to the fast-twitch skeletal muscle with no metabolic stability and rapid fatigue. Analysis of the literature shows that an increase in the rate of oxygen consumption by order of magnitude, due to Frank–Starling law, is observed without any significant changes in the intracellular calcium transients. Therefore, parallel activation of contraction and mitochondrial respiration by calcium ions may play only a minor role in regulation of respiration in the cells. The effective regulation of the respiration under the effect of Frank–Starling law and metabolic stability of the heart are explained by the mechanisms of functional coupling within supramolecular complexes in mitochondria, and at the subcellular level within the intracellular energetic units. Such a complex structural and functional organisation of heart energy metabolism can be described quantitatively by mathematical models. (Mol Cell Biochem **256/257**: 185–199, 2004)

Key words: heart, skeletal muscle, mitochondria, respiration, regulation, mathematical modelling

Introduction

One of remarkable metabolic consequences of structural organization of cardiac cells is an apparent complexity of the regulation of mitochondrial respiration *in vivo*, in contrast to mitochondria isolated from tissue. In *in vitro* experiments, the respiratory control phenomenon by ADP is an undisputed and classical control mechanism of respiration of isolated mitochondria [1, 2]. It is, however, not easy to apply this knowledge for the living muscle cells. The ADP concentration in the cytoplasm, because of its very low value, has never been measured in muscle cells directly, but has usually been cal-

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culated from the creatine kinase reaction kinetics or equilibrium, in dependence on the authors' view on the role of this reaction in cellular energetics [3–6]. Studies of the regulation of respiration in the cells *in vivo*, performed mostly by ^{31}P-NMR method, have given different results for various muscle types. While in skeletal muscles the increased respiration rate during or after the exercise can still be explained by an increase in calculated ADP [7], in heart cells the respiration rate is increased manifold without any significant change in the intracellular levels of phosphocreatine and ATP, and thus, in the 'apparent' cytoplasmic ADP concentration, calculated from creatine kinase equilibrium [8–10]. This basic phenomenon of metabolic stability of the heart, first described by Neely *et al*. [8] and then by Balaban *et al*. [10], has strongly stimulated the research of possible alternative mechanisms of respiration regulation *in vivo* and, by the way, sharply divided the researchers into two camps. On one hand, several groups advocate a theory of parallel activation of muscle contraction and respiration by calcium ions [11–13]. On the other hand, many researches, presented particularly by multiple authors of this volume, still support the more conservative point of view that the metabolic feedback mechanism of regulation of respiration is the major one in the heart cells. This point of view is based on the data showing high degree of structural and functional organization of cardiac cells' energy metabolism. It states that it is the phenomenon of metabolic channelling via energy transfer networks which makes the feedback regulation of respiration extremely efficient and may explain quantitatively the observed metabolic stability of heart during workload changes (see below). The well-established phenomenon of activation of mitochondrial respiration by Ca^{2+} ions [11, 13, 15–18] is taken by this theory to be needed only for maximally activating the Krebs cycle enzymes (and probably also F_oF_1) to avoid unnecessary limitations of substrate supply, at high workloads, for the respiratory chain and maximal activities of oxidative phosphorylation, which all are subjects to the feedback metabolic regulation.

In this review, we summarize the data available in the literature in order to support the latter point of view. The most important argument in favour of the theory of feedback metabolic regulation is the basic phenomenon of cardiac physiology, the Frank–Starling law by which the cardiac work and oxygen consumption can be changed manifold during elevation of left ventricle filling, without any apparent significant changes in the calcium transients in cytoplasm. Under these conditions, metabolic feedback regulation is the only possible major mechanism of regulation of respiration, and the metabolic stability is achieved due to the phenomena of functional coupling, both at the level of supramolecular complexes in mitochondria and within intracellular energetic units at the cellular level.

Respiration rate *vs* workload and metabolic stability of the heart

To the knowledge of the authors of this article, the first experimental investigation of the connection between heart work and respiration was the study by Starling and Visscher entitled: 'The regulation of the energy output of the heart' [19], published in 1926 in *Journal of Physiology*, long before the discovery of the mitochondrial oxidative phosphorylation. In this study the authors showed that both the cardiac work (measured in kg.m), and oxygen consumption increased linearly with the increase of left ventricular diastolic volume [19]. This was the basic physiological observation. Much later, the problem of regulation of mitochondrial respiration in cardiac cells as a serious scientific challenge in molecular and cellular cardiology was reformulated about 25–30 years ago by J.R. Williamson and J.R. Neely [8, 9, 14], two remarkable representatives of founders of experimental cardiology. Neely *et al*. described the linear relationship between cardiac work and the rate of oxygen consumption by isolated perfused rat heart [9], thus confirming the much earlier observations by Starling and Visscher [19], and showed that this may be observed without any changes in the phosphocreatine level [8]. Williamson *et al*. [14] used Neely's working isolated rat heart model and gave the quantitative description of the scale of these changes. In the classical study by the Williamson group [14], different steady state levels of cardiac work were achieved by pumping perfusate at a different rate through the left atrium and the cardiac work was calculated as the product of mean aortic pressure and left ventricular output. In this physiological model, the change of the cardiac work with an increase in the rate of the left ventricle filling (the Frank–Starling phenomenon) caused proportional increase in the oxygen consumption rate about 15–20 times, from 6–12 μmol/g dw/min up to 170 μmol/g dw/min. In the rat heart tissue, the mitochondrial content is close to 70–80 mg/g ww or about 350 mg/g dw [8, 9, 20], giving the observed maximal oxygen consumption rate in the heart *in vivo* and calculated per mg of mitochondrial protein (490 nmol O_2/min/mg) rather close to the value usually found for maximal State 3 respiration of isolated mitochondria at 37°C [20]. The most important result of Williamson *et al*. [14] on the linear dependence of the rate of oxygen consumption on workload is reproduced in a modified form in Fig. 1. Under these conditions, the creatine/phosphocreatine ratio decreased only from 1.7 to 1.0, that means only about 30% decrease in the phosphocreatine content (from about 15 to 12 mM, see below) [14]. This represents a remarkable phenomenon of the metabolic stability in the heart. Later, the phenomenon of metabolic stability was registered also by a ^{31}P-NMR method for smaller interval of workload changes by Balaban *et al*. [10] and confirmed by Wan *et al*.

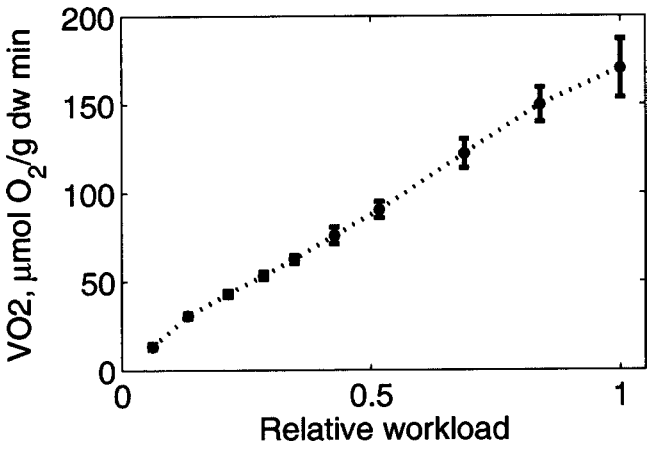

Fig. 1. The dependence of the rate of oxygen consumption by isolated perfused working rat heart on the work regulated according to the law of Frank–Starling. Data from the work of Williamson *et al.* [12]. The maximal workload corresponded to 0.6 kg/m (gdw/min) at the ventricular filling rate 56 ml/min [12].

[21], who used the standard metabolic assays for measurement of ATP and phosphocreatine contents. Figure 2A illustrates this phenomenon on the basis of the latter publication. Figure 2B shows, for comparison, the absence of metabolic stability in fast-twitch skeletal muscle, where the phosphocreatine level always decreases rapidly during the exercise [7].

The phenomenon of the metabolic stability of the heart, together with the Frank–Starling law, is the most important characteristics of the cardiac energy metabolism and physiology. Because of the metabolic stability, the state of fatigue is unknown for the healthy heart.

To explain the regulation of mitochondrial respiration in cardiac cells *in vivo* means to explain qualitatively and quantitatively the observations made by Starling, Visscher, Neely, Williamson and others: the phenomenon of metabolic stability at 20-fold changes in the rate of oxygen consumption linearly which is related to workload increase in accordance with the Frank–Starling law.

In many further studies, numerous researches have subsequently pursued this line of investigations, but very often the experimental technique has been limited only to the convenient use of minor workload changes in Langendorff perfused isolated rat hearts or *in vivo* operated dog hearts stimulated by adrenergetic agonists, sometimes not more than only 2 times [22, 23]. An extreme case is the study in which 20-fold possible workload change was diminished into only 20% changes [23]. These 20% changes are close to the range of experimental errors in physiological experiments, and this type of experimental protocol clearly gives no basis for reasonable conclusions.

Effect of changes of intracellular Ca^{2+} concentration on mitochondrial respiration

The excitation–contraction coupling mechanism involves cyclic changes of the intracellular concentration of free Ca^{2+} ions in cardiac cells, represented usually by discrete localised changes, calcium sparks, in multiple microcompartments in the intracellular space [24–28]. Mitochondria participate actively in this process [15–18]. In connection to the topics of this review, the most important question is whether the excitation-contraction coupling and changes in cytosolic calcium are involved in the regulation of mitochondrial respiration, in parallel with activation of contraction, or not.

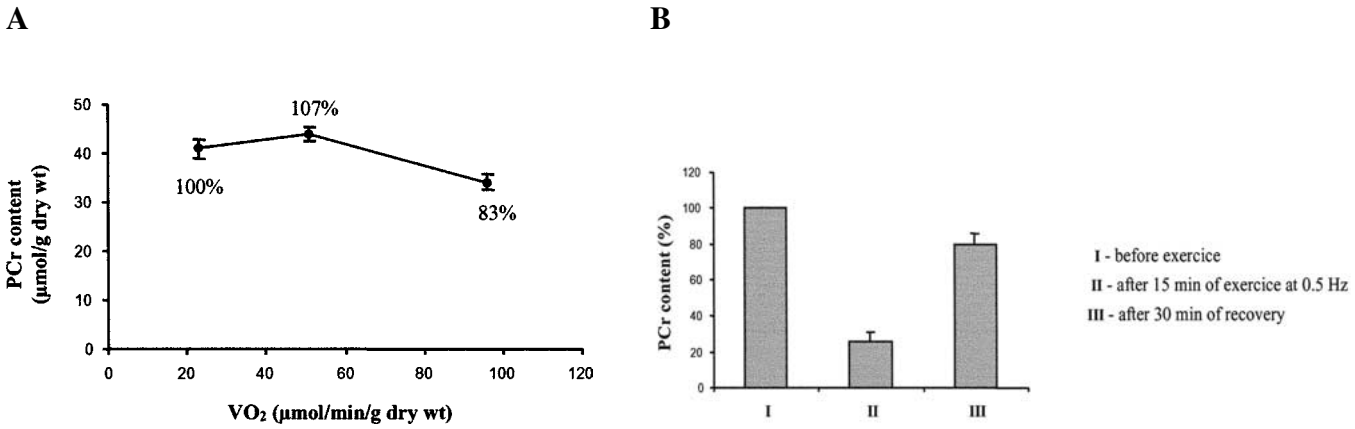

Fig. 2. (A) The remarkable metabolic stability of the heart. Data from Wan *et al.* [21]. (B) The absence of metabolic stability in fast-twitch skeletal muscle. I – before exercise; II – after 15 min of exercise at 0.5 Hz; III – after 30 min of recovery. Data from Kushmerick *et al.* [7].

Parallel activation of contraction and respiration by calcium: pro

The mechanisms of the interplay between mitochondria and cellular calcium are in details described by Jacobson and Duchen in this volume. Among the multiple mechanisms of these interactions are the effects of activation of the mitochondrial dehydrogenases by calcium discovered and described in detail by Hansford, Denton and McCormic [15, 28]. It is clear that rapid uptake of calcium by mitochondria [16, 27] is necessary for activation of the key dehydrogenases of the Krebs cycle and others, usually by the mechanism of increasing the affinity of these enzymes for their substrates [15]. Another enzyme which may be activated by Ca^{2+} ions has been reported to be the F_oF_1 ATPase [17]. Detailed investigations of the effects of calcium on mitochondrial F_oF_1 ATPase, $\Delta\Psi$ and respiration were recently carried out by the Balaban group (reviewed in [13]). These studies have shown that in the isolated and Ca^{2+}-depleted mitochondria, the respiration rate was already relatively high (196.6 $nmolO_2$/min/nmol cyt_a), but was rapidly increased (to 307 $nmolO_2$/min/nmol cyt_a) in response to the addition of calcium into the medium to the final concentration 535 nM, [17, 18]. Similarly, the State 4 rate was increased also by factor of 2 [17]. This increase in respiration was observed in parallel to the increase of production of NADH in mitochondria [17, 18]. Since no uncoupling of oxidative phosphorylation by calcium under these conditions was seen, these observations indicated also the activation of ATP production in mitochondria, but the direct effect of Ca^{2+} on F_0/F_1 remains unclear [17].

Thus, according to the experimental data, available now in the literature, changes in mitochondrial calcium are rapid enough to participate in regulation of respiration, and can increase the respiration rate up to 2 times with an increase of the free cytoplasmic calcium concentration up to 600 nM [17, 18]. Elevation of $[Ca^{2+}]_i$ in this range in resting muscle can be easily achieved after adrenergic activation of the β-receptors by the activating calcium-induced calcium release (CICR) via increasing calcium influx through the slow calcium channel in cardiac cells' plasma membrane [24].

Parallel activation – contra: Frank–Starling law.

From the discussion above it is clear that the regulatory mechanism via activation of mitochondrial enzymes by calcium, assumed by Balaban *et al*. to be the principal one [13], is unable to explain maximally possible 15–20-fold increase in the oxygen consumption under the effect of the law of Frank–Starling in the heart *in vivo.* To understand this discrepancy, the next important and intriguing question is: does, and how much, the $[Ca^{2+}]_i$ changes when the force of contraction and cardiac work are increased in accordance with the Frank–Starling law. The analysis of the available data shows that not too much.

Kentish and Wrzosek studied the changes in force and cytosolic $[Ca^{2+}]_i$ after length changes in isolated rat ventricular trabeculae by micro-injecting of calcium specific probe fura-2 [29]. A step increase in length of the muscle produced a rapid potentiation of twitch force but not the calcium transient [29]. Since direct release of calcium from sarcoplasmic reticulum (SR) into the microdomains close mitochondria, due to their proximity, is a well-known phenomenon [16] and may mask the effects of length changes on calcium transients in cytoplasm, the authors used ryanodine and cyclopiazonic acid to inhibit the release of calcium from SR and its uptake by SR, respectively. While both the twitch and the calcium transients were reduced and prolonged by SR inhibition, the rise of the force after muscle re-lengthening was not affected [29]. The authors concluded that the rapid increase in the twitch force after muscle stretch was owing to length dependent properties of myofibrils, mostly due to increase in myofibrillar Ca sensitivity. Earlier, similar conclusions were made by Allen and Kentish [30]. Very recently, Shimizu *et al*. measured calcium and ventricular pressure transients on isovolumic and ejecting contractions in isolated, blood-perfused physiologically after loaded canine hearts at different left ventricle volumes [31]. While there was an approximately linear relationship between peak pressure and ejecting volume of contractions, as expected by Frank–Starling law, no detectable influence of the volume change on the intracellular calcium transients was observed, and the peak value of $[Ca^{2+}]_i$ was always close to 800 nM [31]. According to the parallel activation theory and experimental data presented by the Balaban group [17, 18], mitochondrial respiration should always proceed with the constant rate rather close to Vmax under these conditions, in contrast to the observation by Williamson *et al*. [14]. Indeed, from the enzyme kinetics point of view, activation of an enzyme by an activator (in this case by calcium) which binds to the enzyme molecule, follows the hyperbolic Langmuir type saturation kinetics [32]. Thus, if the mitochondrial enzymes are activated by Ca^{2+} ions, the respiration rate should follow the same kinetics. In experiments with isolated mitochondria *in vitro*, this type of dependence has been shown by the Balaban group to be true for the range of calcium concentrations from 0–600 nM [17, 18]. The physiological range of changes in the mean cytoplasmic calcium concentration, however, may extend up to 1–3 μM [24], and the calcium concentration in the local areas (calcium sparks) may be even much higher. In these ranges of calcium concentrations, no effects of calcium on respiration rates could be expected. Thus, the kinetics of the action of calcium on mitochondrial enzymes *in vitro* does not explain the experimentally observed linear dependencies of respiration rate on workload *in vivo* reported by the Williamson group [14].

In conclusion, two basic phenomena of the heart physiology – the Frank–Starling law and the related linear dependence of the oxygen consumption on workload – are not consistent with, and even seem to exclude the parallel activation of respiration and contraction by calcium. Therefore, only metabolic feedback regulation mechanism can explain the observed phenomena. In the Frank–Starling mechanism, the increase of sarcomere length and possible increase of the calcium sensitivity of myofibrils result evidently in the increase of number of active cross-bridges and force development at the expense of free energy change in the actomyosin MgATPase reaction coupled to myofibrillar creatine (and adenylate) kinase reactions [33–37]. Usually, the CK-phosphocreatine system is the source of energy for muscular work [35–37]. During cardiac muscle contraction, either ADP or creatine, the phosphate acceptors, need to be rapidly rephosphorylated to maintain the metabolic stability of the heart, and for that they should be resupplied to mitochondria. This metabolic feedback flux is the most plausible regulatory signal for mitochondrial respiration.

This conclusion is in concord with the important results of studies of responses of mitochondrial NADH to the rapid workload changes in heart cells *in vivo* described by Brandes and Bers [38–40]. These authors showed that in response to the sudden increase in the work, the mitochondrial NADH in heart becomes always more oxidized, pointing to the activation of the mitochondrial respiratory chain by the regulatory signal from cytoplasm, most probably by ADP. The first phase of mitochondrial NADH oxidation was always followed by its reduction due to activation of mitochondrial enzymes and increased substrate supply for the respiratory chain [38–40]. The second recovery phase is very consistent with the known effect of Ca ions on mitochondrial dehydrogenases. These experiments strongly suggest the primary role of ADP in regulation of respiration *in vivo*, i.e. the concept of metabolic feedback regulation of respiration [41].

Fiolet *et al*. manipulated the cytoplasmic calcium concentration in cardiomyocytes via Na^+/Ca^{2+} exchanger by changing extracellular cation composition and measured both intracellular calcium concentration (with Indo-1) and respiration rate [42]. They found that a change of extracellular cation composition caused a transient increase both in intracellular calcium concentration and respiration rate [42]. The observed relationship between these two parameters was not changed in the presence of ruthenium red, an inhibitor of mitochondrial calcium uptake [42]. The authors concluded that not the direct effect of calcium on mitochondrial enzymes, but increased energy expenditure stimulated the respiration in their experiments (supporting thus the concept of metabolic feedback regulation). Similar conclusion was made by Khuchua *et al*. in the study of respiration regulation in permeabilized human skeletal muscle fibers [43].

Thus, in all cases the experimental results can be easily explained by metabolic feedback regulation of the respiration.

As mentioned by Jacobson and Duchen in their chapter in this volume, 'cellular calcium signalling seems to underpin almost indecent array of processes'. The mitochondria, by a mechanisms of rapid exchange of calcium with cytoplasm, seem to actively participate in regulation of calcium signaling, the main effects of calcium on mitochondrial function being the control of the level of activity of the Krebs cycle dehydrogenases and permeability state of the inner membrane. Besides, the efficient feedback regulatory signal should never be as harmful as an excess of calcium could be for mitochondria.

According to the terminology of the Metabolic Control Analysis [44], the flux control coefficient over the respiration rate by the processes of metabolic signaling is most probably close to one under the effect of Frank–Starling law, and only for constant left ventricle filling some values could be ascribed to processes of Ca-dependent activation of mitochondrial enzymes.

The organised creatine kinase systems: Different roles in different muscles

The limitations and controversies of the hypothetical mechanism of parallel regulation of respiration and contraction by $[Ca]_i$ described above are easily and logically overcome by metabolic feedback regulation mechanism in the organized metabolic systems. The main candidates for this type of regulation are, as indicated above, the creatine kinase (CK) and the adenylate kinase (AK) systems, in details described in this volume and elsewhere [44–48]. We here summarize only some of the important aspects concerning the regulation of mitochondrial respiration.

It is some kind of scientific myth invented by proponents of the parallel activation theory that the activity of the creatine kinase in cardiac cells is so high that the reaction is always in a rapid equilibrium, rapidly buffers the concentrations of ADP and ATP and therefore can never be regulatory [13, 50]. These statements are very far from reality. The well-known experimental data show the contrary: the heart has almost the lowest CK activity among different muscles, it is lower only in the smooth muscle, and the total creatine kinase activity is 3–7 times higher in the fast-twitch glycolytic muscle than in heart ([51, 52], see also the chapter by Ventura-Clapier in this volume). These data are reproduced in Fig. 3. At the first sight, there is an apparent paradox in these data. For creatine kinase, both the thermodynamics and kinetics of the reaction are not favourable for the phosphocreatine (PCr) synthesis and thus for metabolic stability, they are favourable for ATP synthesis at the expense of phosphocreatine [53]. Nevertheless, the cardiac cells have relatively low creatine kinase activity (Fig. 3), but very high metabolic stability, in particular, very stable level of phosphocreatine due to high steady state rate of its

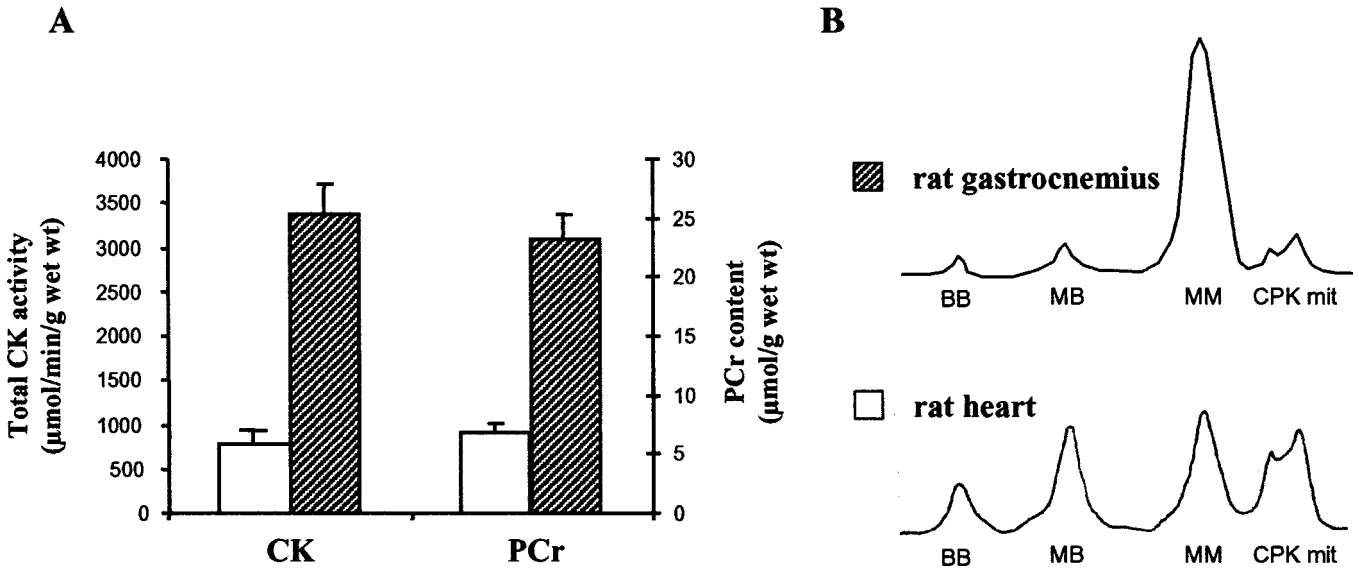

Fig. 3. Tissue specificity of the total creatine kinase activity and phosphocreatine content at rest (A), and the isoenzyme spectra of creatine kinase (B). Data from [51–53].

reproduction in mitochondria (Fig. 2A). Vice versa, the fast-twitch glycolytic muscles have very high creatine kinase activity and high phosphocreatine content at the rest, exceeding those of cardiac cells by a factor of 3–7 (see the chapter by Ventura-Clapier *et al*.), but practically no metabolic stability: during the exercise, the phosphocreatine content decreases rapidly (Fig. 2B), inorganic phosphate increases and the muscle performance diminishes because of the fatigue [7]. Oxidative skeletal muscles, such as *m. soleus* etc. occupy an intermediate position.

The explanation of this apparent controversy is given by the differences of structural and functional organization of the energy metabolism in the cells [51–59]. What changes the functional behaviour of the creatine kinase in mitochondria is its functional coupling to the adenine nucleotide translocator, ANT and thus to the oxidative phosphorylation. Cardiac and skeletal muscles have, first, very different mitochondrial content and their intracellular localization, and second, the different isoenzyme spectra of the creatine kinase, particularly with respect to the mitochondrial creatine kinase, miCK. In the fast twitch skeletal muscle mitochondria occupy only about 2% of the cell volume, they are localised mostly close to the T-tubuls and Z-line [54] and high MM creatine kinase activity is of cytoplasmic origin, miCK being present only in trace amounts (Fig. 3). On the other hand, in cardiac cells mitochondria are localized at the level of A-band of sarcomeres [55, 56], in close proximity of the sarcoplasmic reticulum [34], occupy 30–40% of cell volume [56], and the miCK represents about 40% of the total creatine kinase activity [57]. On the basis of functional and structural studies it has been concluded that in heart cells mitochondria are organized into functional complexes with SR and sarcomeres, intracellular energetic complexes, ICEUs [58–60]. In these highly organized structural units, the communication between mitochondria and energy-consuming structures is carried out by energy transfer and signal transduction networks of CK and adenylate kinase, AK [58, 59]. The intensive and elegant *in vivo* kinetic studies by Dzeja and Terzic have given us the best functional evidence of these networks (see the chapters by Dzeja *et al*., Pucar *et al*. and Selivanov *et al*. in this volume), confirmed by recent ^{31}P-NMR inversion transfer studies performed by the Hoerters group ([61], reviewed by Joubert *et al*. in this volume).

The functional coupling as a general pattern of mechanisms of metabolic control at supramolecular and cellular levels

Functional coupling in supramolecular complexes

In mitochondria, the miCK is bound to the external surface of the inner membrane by cardiolipin in close association to the adenine nucleotide translocator [53, 62–65]. Stoichiometric studies have shown the presence of miCK and ANT in equimolar amounts in cardiac and skeletal muscle mitochondria (measured by specific binding of ADP analog; [66]), and the binding of the inhibitory monoclonal antibodies to miCK inactivates the ANT [67]. The architecture of the complexes of these proteins may be different in different locations: di-

mer to dimer, or octamer of miCK in complex with a cluster of ANT [36]. Dieter Brdiczka has described the possible localisation of these complexes in the contact sites (see the chapter by Vysokikh and Brdiczka in this volume). The Wallimann group and several other authors favour the point of view that the normal state of the miCK in heart mitochondria is an octameric form [36, 37], but the question is still not completely clear [68]. In any case, in heart mitochondria the maximal activity of miCK in direction of PCr production is exactly equal to the maximal rate of the ATP production in the oxidative phosphorylation (close to 1 μmol/mg/min at 30°C) [53, 69, 70]. Thus, there is no excess capacity of the miCK, as assumed by Balaban [13] and Korzeniewski [50], and in fact there is no need for it. Because of very precise localisation of miCK with respect to ANT, the ATP is directly channelled by ANT from matrix into microcompartment ('gap') between these proteins [53, 65, 69–71] and quickly saturates the active site of miCK. ADP produced simultaneously with PCr in the creatine kinase reaction is released also into this microcompartment where a large part of it is rapidly taken up by ANT into matrix in exchange for new quantities of ATP. The local ratio of ATP/ADP available for miCK in this gap is always much higher than that in the medium and forces, both thermodynamically and kinetically, the miCK reaction to proceed in direction of PCr synthesis [53, 65, 69–71]. Channeling of ATP and ADP into this microcompartment and their turnover between miCK, ANT and oxidative phosphorylation represents the mechanism of functional coupling between these systems [69, 72], which amplifies the metabolic signals from cytoplasm into mitochondrial matrix, providing thus the basis for the metabolic stability of the heart [36–38, 69–72].

This coupled ANT-miCK system is an excellent example of the general phenomenon of functional coupling in supramolecular complexes of enzymes, transporters (and sometimes channels) , which can be described by following formulae:

Functional coupling = Metabolic channelling + microcompartmentation,

indicating the synergistic and amplifying effects of both factors: the vectorial transfer of metabolites and their accumulation in small intracellular spaces.

Fossel and Hoefeler showed in an interesting model experiments with creatine kinase and hexokinase covalently attached to Sepharose beads that the functional coupling of these two enzymes by metabolic channeling is observed at an average distance of the order of 10 nm between enzymes [73].

It is important to note that the functional coupling is a dynamic phenomenon in a sense that there is also a leak from a microcompartment by simple but restricted diffusion into the medium (cytoplasm). If for some reason the second enzyme connected to the microcompartment is not active (for example, if miCK is inactivated by genetic modification or CK substrate creatine is absent) the microcompartment becomes rapidly saturated and the system behaves as a single enzyme (or transporter) supplying its product to the medium. In this case, the adenylate kinase system can take over the role of energy transfer and signal transmission. On the other hand, if both components are active and functional coupling is operating, the rate of the second reaction will be almost linearly related to the rate of the first one [74]. In the case of coupled ANT and miCK, the rate of phosphocreatine production is linearly related to the rate of oxidative phosphorylation [74, 75]. Because of this kind of coupling, the creatine kinase reaction in mitochondria is clearly out of equilibrium, shifted in the direction of phosphocreatine synthesis, the higher the rate of oxidative phosphorylation, the farther the reaction from equilibrium [5, 6, 71]. If the relationships between the rates of oxidative phosphorylation and mitochondrial phosphocreatine production are underestimated or ignored, one may fail to observe functional coupling between miCK and ANT in heart mitochondria. For example, when the mitochondria with very low respiratory activities were used, the functional coupling could not be activated and therefore detected [76].

Both the creatine kinase and adenylate kinase systems of energy transfer are based on the mechanisms of the functional coupling in different subcellular sites, in mitochondria, myofibrils and at the subcellular membranes [77]. Within the creatine kinase system, the functional coupling means that different CK isoenzymes function in two different directions out of equilibrium, the mi-CK in the direction of PCr synthesis and the MM-CK (in myofibrils, SR and sarcolemma) in the direction of PCr utilization for the local regeneration of ATP near ATPases [5, 6]. This was shown first by using the methods of mathematical modelling [5, 6, 53], and then verified experimentally by using the inversion transfer methods of ^{31}P-NMR in hearts *in vivo* [61]. The importance of the functional coupling of miCK with the ANT for metabolic stability of the heart was recently verified by Spindler *et al.* [78] in a study of isolated perfused transgenic hearts with the deficiency of the sarcomeric form of mitochondrial creatine kinase: in these hearts the PCr/ATP ratio was considerably decreased.

In the bulk water phase, in the cytoplasm the MM-creatine kinase reaction is coupled to the glycolytic system, which ensures use of ATP produced in phosphoglycerate kinase and pyruvate kinase reactions for creatine phosphorylation and production of PCr. The lack of direct structural connection between cytoplasmic MM-CK and glycolytic system results in the lack of metabolic channeling. In these reactions the creatine kinase is operational in the state of quasi-equilibrium, and the synthesis of PCr is a result of constant removal of ADP by the glycolytic system at an almost unchanged level of ATP [79]. As a result, the steady state ATP concentrations exceed by orders of magnitude that of ADP, this leading to a continuous shift of the creatine kinase equilibrium position. It was also revealed that interaction between glycolytic system and oxidative phosphorylation can be effectively regulated by

creatine kinase system: high PCr to creatine ratio reached in the cytoplasm due to oxidative phosphorylation inhibits the overall glycolytic flux due to limited availability of ADP [79]. Due to the quasi-equilibrium kinetics of the MM-creatine kinase and because of the absence of direct metabolic channeling of the ATP in cytoplasm, the PCr production coupled to glycolytic system is rather a slow process, since it is the kinetically and thermodynamically unfavourable direction of the CK reaction. (It should be noted here that this consideration concerns only the glycolytic enzymes homogeneously distributed in the cytoplasmic bulk water phase). This, although compensated by much higher total activity of MM-creatine kinase and glycolytic enzymes than in cardiac cells, results in inability of the fast twitch muscle cells to maintain the constant phosphocreatine levels during continuous burst of tetanic contractions of high intensity. In other words, the developing metabolic instability progresses into fatigue. Then, the period of rest is needed during which, on one hand, the oxygen debt is paid back due to activation of mitochondrial respiration, and, on the other hand, the cellular sources of PCr are replenished due to PCr synthesis predominantly via mi-CK coupled to ANT, until the high PCr/creatine ratio typical for the resting state is achieved [80–83].

In the skeletal muscle mitochondria the miCK is, like in the myocardium, tightly coupled to ANT [79] and the respiration rate in the skeletal muscle cells is controlled by the creatine kinase system and PCr/creatine ratio [80–82]. However, during contractile activity this oxidative mechanism of phosphocreatine production is of minor importance because of low content of mitochondria and, respectively, miCK in the cells. On the other hand, at the other end of the system, the PCr is always effectively used for providing ATP near ATPases via coupled MM-creatine kinase, and here the enzyme is functioning in direction of rephosphorylation of ADP at the expense of PCr, according to the kinetics and thermodynamics of the reactions and structural organization of myofibrils, and leading to the mechanism of functional coupling between MM-creatine kinase and myofibrillar ATPases (see the chapter by Ventura Clapier *et al*., in this volume).

Figure 4 illustrates schematically the differences in the organisation of the energy metabolism in the two extreme muscle types: cardiac muscle and fast-twitch skeletal muscle. Comparison of these muscles shows very clearly the importance of the structural organization of the energy metabolism for the efficiency of its regulation. What makes the creatine kinase system in heart cells (with its low, in comparison with skeletal muscles, activity) so efficient that it is capable to maintain the metabolic stability and at the same time to control effectively the mitochondrial respiration rate, is the very high level of structural and functional organization of energy metabolism, and the mechanism of functional coupling between miCK and ANT. For the cell, this is the most easiest way to multiply the metabolic efficiency at no cost, just by putting the right enzyme at the right place. This simple but important principle of organization of enzyme systems gives rise to the phenomena of metabolic channeling and microcompartmentation, both in interaction resulting in the functional coupling on enzymes and transporters. In fast-twitch skeletal muscle, the high activity of glycolytic enzymes and MM-creatine kinase cannot compensate for the lack of this

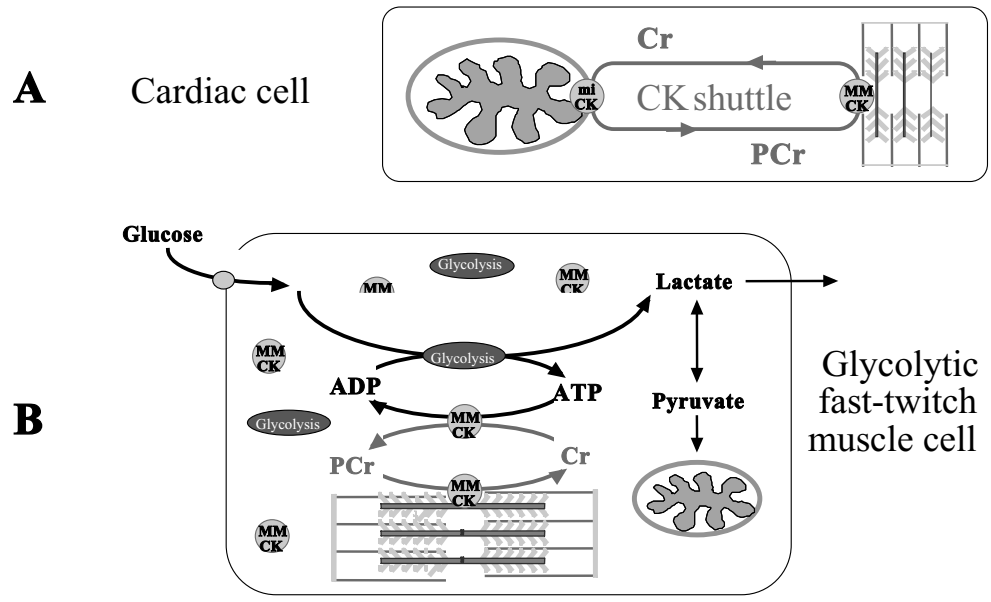

Fig. 4. The schematic presentation of the differences between the systems of the energy metabolism of cardiac (A) and fast-twitch skeletal muscle (B). For explanation, see text.

organization. Oxidative skeletal muscles occupy an intermediate positions between these two extreme cases [84].

The mechanism of the functional coupling may be the basic one in the organised metabolic systems in all cells. Some of the well established examples are described in Chapter 1 of this volume. It is valid also for the glycolytic systems which are compartmentalized and even structurally associated either in myofibrils and at the subcellular membranes, giving rise to similar phenomena of the functional coupling and metabolic channeling (see chapter by Ventura-Clapier *et al*. in this volume) and to the phenomenon of 'glycolytic ATP' [85, 86].

It should be emphasised that the equally important reason of the metabolic stability of the heart, together with functional coupling between miCK and ANT, is the high content of mitochondria specifically organised into functional complexes with myofibrils and sarcoplasmic reticulum (see the next section).

Functional coupling at the subcellular level – intracellular energetic complexes

Investigations of the kinetics of regulation of mitochondrial respiration in the permeabilized cardiac cells by endogenous ADP have shown that ADP generated in the cells by MgATPases may be channelled preferably to mitochondria before being released into the medium [59]. This is the kinetic evidence of the functional coupling at the subcellular level between different organelles and cellular structures. Already in 1988 Kümmel explained these effects by the spatial proximity of Ca,MgATPases (mostly sarcoplasmic reticulum) and mitochondria [87]. Furthermore, it has been shown that the endogenous ATP produced in mitochondria is much more effective substrate for the CaATPase of sarcoplasmic reticulum than the exogenous one in the permeabilized cardiac fibers

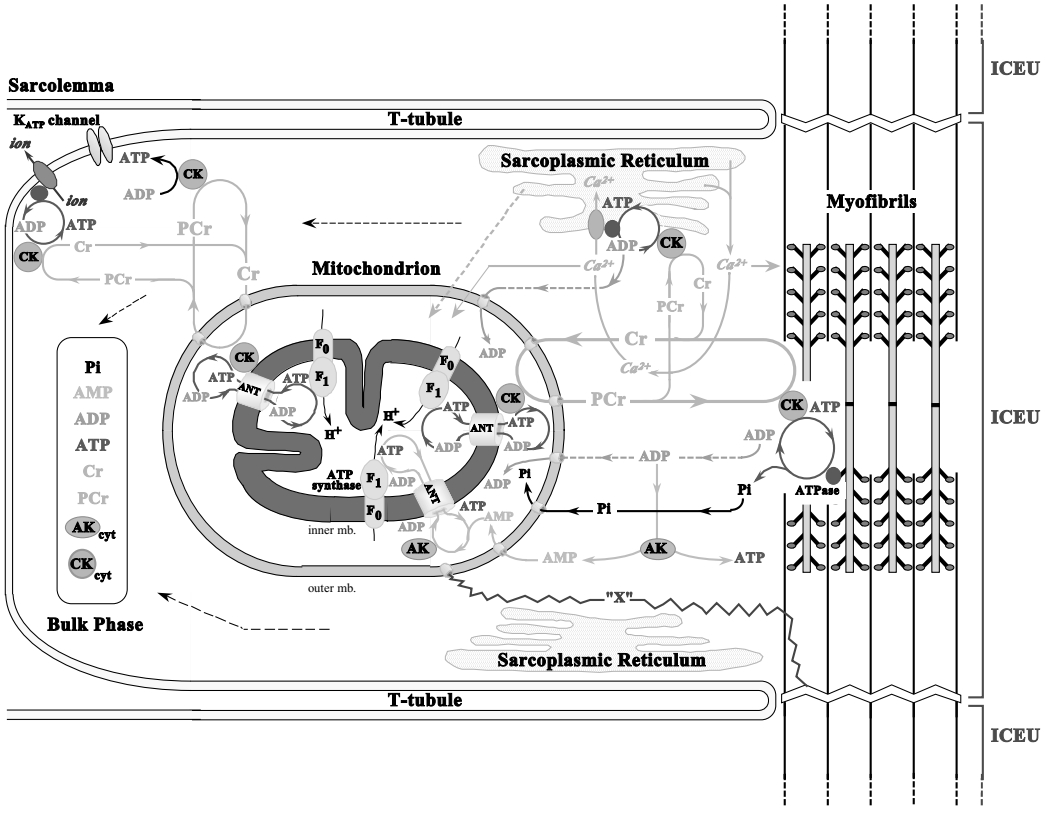

Fig. 5. Schematic presentation illustrating the functional Intracellular Energetic Units (ICEUs) in the cardiac cell. By interaction with cytoskeletal elements [60], the mitochondria and sarcoplasmic reticulum (SR) are precisely fixed with respect to the structure of sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Calcium is released from SR into the space in ICEU in the vicinity of mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within ICEU do not equilibrate rapidly with adenine nucleotides in the bulk water phase. The mitochondria, SR and MgATPase of myofibrils and ATP sensitive systems in sarcolemma are interconnected by metabolic channeling of reaction intermediates and energy transfer within ICEU by the creatine kinase-phosphocreatine and myokinase systems. The protein factors (still unknown and marked as 'X'), most probably connected to cytoskeleton, fix the position of mitochondria and probably also controls the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within ICEU and bulk water phase may be connected by some more rapidly diffusing metabolites as Cr-PCr. Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, Pi or PCr) or/and synchronized release of calcium during excitation-contraction coupling process.

[88]. The studies of these effects by using confocal microscopy and methods of mathematical modeling have led to the conclusion of the heterogeneity of ADP and ATP diffusion and rather strong restrictions of this diffusion in some areas of the cell, in accordance with the pulsed gradient ^{31}P-NMR [89–91]. In general, these studies have led to an important conclusion of the unitary pattern of the organisation of the cardiac (and oxidative skeletal muscle) cell energy metabolism, to the theory of the intracellular energetic units in the muscle cells [58–60]. In accordance with theoretical considerations described in Chapter 1 by Ovadi and Saks in this volume, it has been concluded that mitochondria in oxidative muscle cells are included into the functional complexes with sarcomeres and sarcoplasmic reticulum, and all of them form together the intracellular energetic units, ICEUs [58]. That means that all processes of energy metabolism may take place in a small space of the dimensions of several μm, a macrocompartment, the space occupied by ICEUs, and the energy metabolism of the cell is the result of synchronised functioning of these repeating metabolic units. Inside the ICEUs, multiple microcompartments may exist. Figure 5 illustrates the possible structure of ICEUs in cardiac cells. The microcompartments within ICEUs are formed on the basis of the local restriction of the diffusion of adenine nucleotides due to the specific structural organization of the cell ([89], see the chapter by Vendelin *et al*. in this volume). The ATP in the bulk water phase of the cell represents most obviously some important reserve of the high energy compounds, and due to the heterogeneity of diffusion and its local restrictions, this ATP may be relatively slowly mixed with metabolically important ATP and ADP pools in these microcompartments, which take part in the functional coupling (see the definition of compartmentation in Chapter 1).

The diffusion of ADP (and ATP) may be restricted inside the ICEUs also at the level of mitochondrial outer membrane due to the interaction of some cytoskeletal proteins with the VDAC. Hypothetically, this interaction may involve the interaction of cytosolic (cytoskeletal) proteins with the unusually long loop of VDAC molecule facing the cytosol (see the chapter by Colombini in this volume).

The local restrictions of the diffusion of ATP and ADP [89], and the necessity of maintaining high local values of the phosphorylation potential in microcompartments near all ATPase, especially near the reversible ion pumps [48] under conditions of high energy fluxes explain the existence of the organized creatine kinase and adenylate kinase networks of energy transfer and feedback signalling, by now well and in great details described by the methods of the *in vivo* kinetic studies ([46, 47], see the chapter by Joubert *et al*. in this volume).

The impressive amount of fundamental work carried out by the laboratory of Be Wieringa in Niemingen, The Netherlands, on the genetic modification of the creatine and adenylate kinases has given us firm evidence of the importance of this system: the 'knock-out' of the creatine kinase and adenylate kinase genes results in very significant adaptive changes in the cells to compensate for the loss of this important system, causing structural remodeling of the cells, as described in details in several chapters of this volume. The excellent series of studies from the Terzic and Dzeja groups have shown that any modification of the creatine kinase or adenylate kinase isoenzymes will strongly modify the feedback signal within the network of the phosphotransferase reactions (reviewed in this volume).

The concept of the ICEUs is most probably valid for at least several types of the cells, e.g. for cardiac and oxidative skeletal muscle cells with very high degree of structural organization. This is in full agreement of the data by Collins *et al*. who have shown that in many cells mitochondria are morphologically and functionally heterogeneous and unconnected [92]. There is a clear experimental evidence for this conclusion: destruction of the ICEUs by proteolytic treatment results in liberation of spherical mitochondria easily visible by confocal fluorescent imaging [89].

The cells with the unitary organization of energy metabolism and with specific structures of ICEUs may be very attractive objects for the application of the methods of fractal analysis described in this volume by Aon, O'Rourke and Cortassa. In the fast-twitch skeletal muscle cells, because of the very low content of mitochondria, ICEUs if they exist, they may have a very different structure, since there may still be some important interactions between mitochondria and sarcoplasmic reticulum.

It is not excluded that in some other cells, the mitochondrial network may be much more dynamic and subject to the structural changes in time (see the chapters by van Blieck and Skulachev in this volume). Nevertheless, the concepts of functional coupling (which may be also a dynamic phenomenon) could be helpful for analyzing the regulation of energy metabolism and mitochondrial function in all types of the cells.

Quantitative description of the metabolic stability of the heart by mathematical models based on the mechanism of functional coupling.

Remarkably, the mathematical model of the compartmentalized energy transfer gives reasonable quantitative description of the regulation of the rate of mitochondrial respiration *in vivo* and the phenomenon of metabolic stability of the heart [5, 93]. In Fig. 6 we illustrate some of the useful information obtained by these models which are described in full details elsewhere [93].

The principal basis of the models of compartmentalized energy transfer is the concept of the ICEUs. The spatially inhomogeneous reaction-diffusion models of energy transfer consider the reactions in three main compartments: the myofibril together with the myoplasm, the mitochondrial inter-

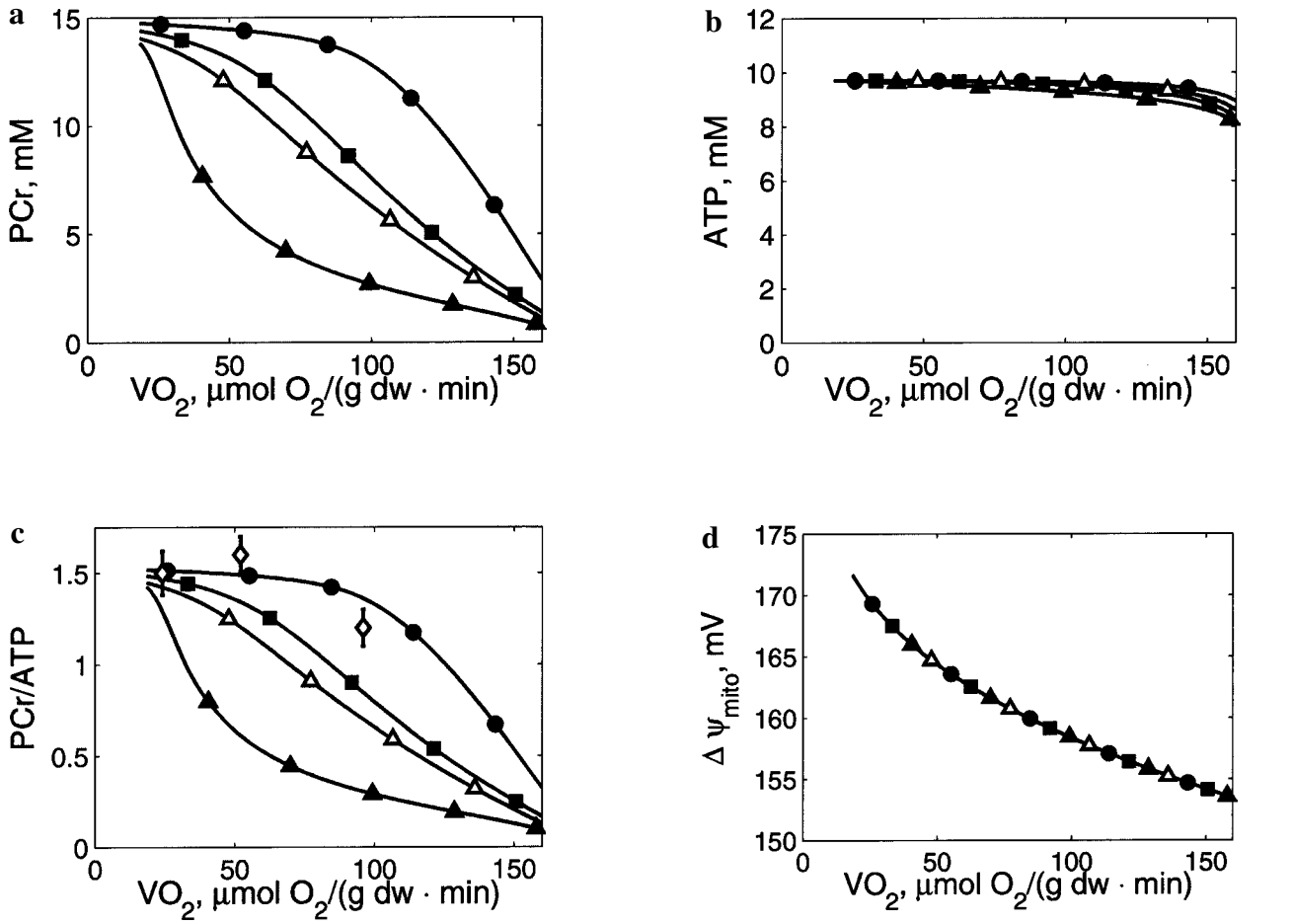

Fig. 6. Mathematical modeling of the metabolic stability of the heart. The simulations were performed using compartmentalized intracellular energy transfer model [93]. In the plot, the calculated average concentrations of PCr (subplot A), ATP (subplot B), and PCr-to-ATP (subplot C) over cardiac cycle as a functions of VO2 are shown. The subplot D shows calculated mitochondrial membrane potential. The decrease in $\Delta\Psi$ with increase of the workload and of the rate of oxygen consumption corresponds to the experimental observation by Wan *et al*. [21]. Respiration rate was changed by the alteration of workload as in [93]. The simulations were performed for the following conditions. Curve 1 (with solid triangles) – there is no miCK activity but 5 times increased activity of MMCK, permeability of the outer membrane is limited. Curve 2 (with open triangles) – there is no miCK activity but 5 times increased activity of MMCK and there is no diffusion restriction on mitochondrial outer membrane for ATP and ADP. These cases correspond to the assumption of Balaban and Korzeniewski [13, 50] of the presence of very high creatine kinase without any coupling; no metabolic stability is achieved. Curve 3 (with solid squares) – activities of miCK and MMCK as in real cells, there is a diffusion restriction on mitochondrial outer membrane for ATP and ADP but miCK and ANT are not coupled. Curve 4 (with solid dots) – activities of miCK and MMCK as in real cells and miCK functionally coupled to ANT, and there is a diffusion restriction on mitochondrial outer membrane for ATP and ADP. Note that only in the case of physiological distribution of CK activities between isoforms and active functional coupling between ANT and MiCK, the metabolic stability is observed and the theoretical curve fits with the experimental points (open rhombs) described in Fig. 2A for isolated working heart preparation.

membrane (IM) space, and the mitochondrial inner membrane-matrix space. This corresponds to the main components of the intracellular energetic units, ICEUs (58, 89]. The metabolites described by the model in the myofibrils and IM space are ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr), and Pi. All these metabolites diffuse between the cytosolic and IM compartments, where the metabolites are involved in the creatine kinase (CK) and adenylate kinase (AK) reactions. In addition, the ATP is hydrolysed in the myofibrils and by the sarcoplasmic reticulum. In the IM space the mitochondrial CK reaction is coupled to the adenine nucleotide translocase (ANT); the coupling is moderated by a diffusional leak of the intermediates. The metabolites described by the model in the matrix compartment and in the inner membrane are NADH, coenzyme Q, cytochrome c, protons, ATP, ADP, and Pi. Three coupled reactions representing the production of proton-motive force by complexes I, III, and IV are included in the model, as originally described [12, 93]. Proton-motive force is consumed by ATP synthase and membrane leak. The ANT rate is considered to depend on membrane potential. Pi is transported

by a phosphate carrier. The description of respiratory chain processes was adapted from model by Korzeniewski [12, 93].

Figure 6 shows the calculated average concentrations of phosphocreatine, PCr (A), ATP (B), and the PCr-to-ATP ratios (C) and calculated mitochondrial membrane potential as functions of VO2. The simulations were performed for the non-restricted and restricted ATP, ADP and AMT diffusion through mitochondrial outer membrane for different hypothetical or real conditions: (1) no miCK in mitochondria but 5 times increased activity of MM creatine kinase in cytoplasmic space (curves 1 and 2); (2) the miCK and MM activities as in real cells, but no coupling between miCK and ANT (curve 3); (3) the miCK activities as in real cells, and miCK is functionally coupled to ANT (curve 4). The ATP concentration was always stable (Fig. 6A). Curve 2 corresponds to simulations performed with ATP, ADP and AMT permeability increased by 100 times if compared with the original model parameters. The curves 1, 3 and 4 correspond to the restricted diffusion of adenine nucleotides through the outer mitochondrial membrane. The results of this modelling show that the metabolic stability is observed only for the case of functional coupling between miCK and ANT and restricted outer membrane permeability (curve 4). In this case about 90% of energy flux out of mitochondria is represented by the flux of PCr [5, 93]. No stability is seen for very high MM creatine kinase activity in cytoplasm and no miCK (curves 1). This situation corresponds to the hypothetical one assumed *a priori* by Korzeniewski and Balaban [13, 50]. The PCr levels are more stable but still do not correspond to the experimental observations (see Fig. 6C) when in this hypothetical situation the outer membrane permeability is decreased (curves 2), or in the real situation with miCK in the intermembrane space of mitochondria but no functional coupling. In all these cases, the calculated levels of phosphocreatine decrease rapidly with an increase of workload. The theoretical curve fit the experimental results (open rhombs) only for the functionally coupled miCK (curve 4). The mitochondrial membrane potential changes always in the same way and is related to the rate of oxygen consumption only. It is the metabolic stability which is most sensitive to the mechanism of functional coupling of mitochondrial creatine kinase, and thus to the metabolic feedback signaling. In all these calculations, the Vmax for the respiration was maximal (see above) and constant.

Thus, the experimental data on metabolic stability are quantitatively explained by the phenomenon of the functional coupling within the ICEUs. Earlier we have shown that this explains also the linear relationship between the rate of oxygen consumption by rat heart and cardiac work [93]. The feedback metabolic signal to mitochondria is represented by the cyclic changes of ADP with amplitudes depending on the workload and strongly amplified by the functional coupling phenomena, as well as by changes in other metabolites, including inorganic phosphate [93].

Conclusions

All data available at present time in the literature favor the theory of metabolic feedback regulation of mitochondrial respiration *in vivo*. The alternative theory of parallel regulation of mitochondrial respiration and muscular contraction by calcium ions is not consistent with the basic phenomena of cardiac physiology – the Frank–Starling mechanism and linear dependence on the rate of oxygen consumption on cardiac work, when no changes in the intracellular calcium transients are observed. The theory of organised metabolic systems explains quantitatively the metabolic stability of the heart and changes in the cellular respiration related to the regulation of cardiac work in accordance with the law of Frank–Starling. The best explanation of the experimental data obtained by the use of the permeabilized cell technique is given by the unitary theory of structural and functional organisation of the cellular energy metabolism – by the theory of intracellular energetic units in oxidative muscle cells. In all these systems the central mechanism of their performance is the functional coupling. The answer to a usual question of somehow confused students: why the cells need this complex and complicated system instead of easy-to-understand homogenous one, is simple. The very high degree of structural and functional organisation of energy metabolism is needed for effective regulation, at the lowest possible cost and in the most reliable manner, of the function of organs upon which depends the life of the body and which should work without any errors for many decades. For that, the cardiac (and oxidative skeletal muscle) cells are made as good modern machines, where all details are precisely fitted into their proper places, but not as out-of-time chemical reactors where everything is mixed up and the function is based upon random collisions of molecules of reactants (the analogy proposed by Bernard Guérin, Bordeaux, France). Regulation of energetics is the function of metabolic feedback systems of signaling, in strict dependence on the amount of the work done by the muscle. In this system, the central mechanism is the functional coupling of different enzymes and transporters, which increases the local metabolic turnover of adenine nucleotides and thus amplifies any metabolic signal coming from the cytoplasm.

Philosophically speaking, the reductionism – an attempt to explain the *in vivo* data exclusively on the basis of *in vitro* experiments – fails to explain the mechanisms of cellular bioenergetics. What is lacking in this explanation is the structural and functional organization of the cell *in vivo*.

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