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Organization of energy transfer and feedback regulation in oxidative muscle cells

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V Seppet, E.K., Eimre, M., Anmann, T., Seppet, E., Piirsoo, A., Peet, N., Paju, K., Guzun, R., Beraud, N., Pelloux, S., Tourneur, Y., Kuznetsov, A.V., Käämbre, T., Sikk, P., Saks, V.A. Structure-function relationships in regulation of the energy transfer between mitochondria and ATPases in cardiac cells. Exp. Clin. Cardiol. 11: 189–194, 2006.

VI Eimre, M., Paju, K., Pelloux, S., Beraud, N., Roosimaa, M., Kadaja, L., Gruno, M., Peet, N., Orlova, E., Remmelkoor, R., Piirsoo, A., Saks, V., Seppet, E. Distinct organization of energy metabolism in HL-1 cardiac cell line and cardiomyocytes. Biochimica et Biophysica Acta (BBA) – Bioenergetics, 1777: 514–524, 2008. doi: 10.1016/j.bbabio.2008.03.019

My contribution to original papers:

Paper I
Assessment of kinetics of MgCaATPase activity in the skinned cardiac fibers and determination of ADP concentration in the reaction medium for elaborating and testing the mathematical models of energy transfer.

Paper II
Determination of the activities of adenylate kinase (AK) and creatine kinase (CK) in heart atrial homogenates. Determination of competition between mitochondria and PK + PEP system and between MM-CK and PK + PEP system for

ADP produced in ATPase reactions in skinned heart atrial fibers in order to demonstrate that there exists direct channeling of ADP from ATPases to mitochondria and coupling between MM-CK and ATPases in human heart atrial cells, similarly to that in oxidative muscles of rat and mice. Writing of the Methods and Results sections of the paper.

Paper III
Measuring of the kinetics of ADP production in dependence of [ATP] in conditions of absence and presence of oxidative phosphorylation in skinned cardiac fibers at different [Ca^{2+}]. Calculating from these data the flux of endogenous ADP channeled from ATPases directly into mitochondria. These data allowed to reach one of the main conclusions that endogenous ADP flux, channeled directly from ATPases to mitochondria significantly decreases in a course of Ca^{2+} induced hypercontraction. Writing of the Methods and Results section of the paper.

Paper IV
Determination of the activities of AK, CK, and assessment of the CK isoenzyme distribution in homogenates of *musculus gluteus medius* (MGM). I have analysed all experimental data and made conclusions that energy metabolism in MGM cells is organized similarly to that in oxidative muscles of rat and mouse – in the form of the intracellular energetic units (ICEUs) and that pathogenesis of hip osteoarthritis involves disintegration of these units. I have participated in writing of the paper (Methods, Results, and part of Discussion sections).

Paper V
I have provided experimental data demonstrating the relationships between the direct channeling of ADP from ATPases to mitochondria and the mean sarcomere length altered by the free [Ca^{2+}] in skinned fibers of rat heart.

Paper VI
Preparation of isolated cardiomyocytes. Determination of the flux of ADP produced in ATPase reactions, direct channeling of ADP from ATPases to mitochondria and coupling of cytosolic CK isoforms to ATPases in cardiomyocytes and HL-1 cells. Determination of activities and isoform profile of kinases in cardiomyocytes and HL-1 cells. I have analysed all experimental data and made conclusion that differently from cardiomyocytes where mitochondria and CaMgATPases are organized into ICEUs which ensure effective energy transfer and feedback signaling between these structures via specialized pathways mediated by CK and AK isoforms and direct adenine nucleotide channelling, these units do not exist in HL-1 cells due to less organized energy metabolism. For the manuscript, I have prepared the Figures and large part of the text including the Methods, Results and Discussion sections.

ABBREVIATIONS

Acetyl-CoA	acetyl coenzyme A
AF	atrial fibrillation
ADP	adenosine 5′-diphosphate
ATPase	adenosine triphosphatase
AK	adenylate kinase
AMP	adenosine 5′-monophosphate
AMPK	AMP activated protein kinase
ANT	adenine nucleotide translocase
AP_5A	diadenosine pentaphosphate
ATP	adenosine 5′-triphosphate
ATR	atractyloside
BB-CK	brain type creatine kinase
PBS	phosphate-buffered saline
BSA	bovine serum albumin
CK	creatine kinase
COX	cytochrome oxidase
Cr	creatine
Cyt	cytochrome
DHPR	dihydropyridine receptors
DTT	dithiothreitol
FAD	flavin adenine dinucleotide
$FADH_2$	1, 5-dihydro-flavin adenine dinucleotide
FCCP	carbonylcyanide-p-trifluorometoxy-phenylhydrazone
G6PDH	glucose-6-phosphate dehydrogenase
GTP	guanosine triphosphate
HF	heart failure
HK	hexokinase
ICEU	intracellular energetic unit
IgG	immunoglobulin G
IMS	intermembrane space
LDH	lactate dehydrogenase
MDX	dystrophin knockout
mi-CK	mitochondrial creatine kinase
MGM	*musculus gluteus medius*
MHC	myosin heavy chain
MM-CK	muscle type creatine kinase
MOM	mitochondrial outer membrane
mtDNA	mitochondrial DNA
NAD	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
OA	osteoarthritis
OXPHOS	oxidative phosphorylation
PCr	phosphocreatine

PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PEP	phosphoenol pyruvate
PK	pyruvate kinase
PLN	phospholamban
^{31}P-NMR	phosphorus-31 nuclear magnetic resonance
PTP	permeability transition pore
RyR	ryanodine receptors
SERCA	sarcoplasmic reticulum Ca^{2+} -ATPase
SDH	succinate dehydrogenase
SR	sarcoplasmic reticulum
SL	sarcolemma
TnC	troponin C
VDAC	voltage-dependent anion channel

INTRODUCTION

In striated muscles, ATP for contractions is produced in the systems of glycolysis and oxidative phosphorylation (OXPHOS). Depending on which of the mentioned systems prevails the muscles can be divided into two major classes: the glycolytic muscles (e.g. *white m. gastrocnemius, m. extensor digitorum longus*) and oxidative muscles (e.g. myocardium*, m. soleus*), with a variety of subtypes between these two types (Burke et al., 1971, Peter et al., 1972). The glycolytic muscle cells are characterized by high activity of glycolytic enzymes, large glucogen depositions and low lipid content (Dubowitz, 1985, Howald et al., 1985). The oxidative muscles are rich of capillaries and mitochondria and express high respiratory activity (Andersen, 1975, Kiessling et al., 1974).

The studies on skinned muscle fibers have revealed that the mitochondria in glycolytic skeletal muscle cells express much higher apparent affinity to adenosine diphosphate (ADP) compared to mitochondria in oxidative muscle cells. At the same time, the mitochondria isolated from both types of muscles exhibit similarly high affinity to ADP, comparable to that in skinned fibers of glycolytic muscles (Kuznetsov et al., 1996, Veksler et al., 1995). These findings suggest that OXPHOS is differently regulated in situ, i.e. in conditions when mitochondria can interact with other intracellular structures, and in vitro, in isolated mitochondria, and that intracellular regulation of OXPHOS must be arranged in a muscle type-specific manner.

Recently, it has been hypothesized that in oxidative muscle cells the mitochondria and ATPases form tight complexes, termed as the intracellular energetic units, ICEUs (Saks et al., 2001, Seppet et al., 2001). These complexes compartmentalize part of cellular adenine nucleotides for being used in specialized phosphotransfer networks (Saks et al., 2001, Seppet et al., 2001, Kaasik et al., 2001, Weiss and Korge, 2001). The important feature of these networks is that they ensure effective stimulation of OXPHOS without significant changes in cytosolic adenine nucleotide and phosphocreatine (PCr) contents, a condition termed as metabolic stability (Neely et al., 1972).

At present the structure and molecular basis of the ICEUs are unclear. Direct channeling of adenosine phosphates between organelles identified in the recent experiments indicates that diffusion of adenosine phosphates is limited in cardiac cells due to very specific intracellular structural organization. However, the distribution pattern of diffusion restrictions and molecular nature of the intracellular structures responsible for limited diffusion need to be defined.

In the present study, the different patterns of diffusion restriction distribution for adenine nucleotides are assessed by comparing the results of experimental kinetic measurements with the solutions of alternative mathematical models. Observations that in oxidative muscles disorganization of cellular structure, caused by protease treatment or deficiency of cytoskeletal proteins, leads to increased mitochondrial affinity to ADP in regulation of respiration suggests an important role of regular cellular structure in linking mitochondria and ATPase within one complex (Kay et al., 1997b). The current study further addresses the

structure-function relationships in cardiac cells by comparing the mechanisms of regulation of mitochondrial function in normal cardiomyocytes and cultured cardiac HL-1 cell line, the latter characterized with completely different structure compared to cardiomyocytes, and by inducing structural alterations in the ICEUs by increasing free [Ca^{2+}] in the cytoplasm of cardiomyocytes. It also asks as to whether the ICEUs exist in human muscle cells and are altered under various pathological states.

REVIEW OF LITERATURE

1. Main processes of production and consumption of ATP in oxidative muscle cells

There are specific carriers that transport glucose, lactate and fatty acids into the oxidative muscle cells (Halestrap and Price, 1999, Joost and Thorens, 2001, Koonen et al., 2005). In the cytosol, glucose and lactate are converted into pyruvate, which can be imported into mitochondria and converted to acetyl-CoA via pyruvate dehydrogenase (PDH). Similarly, the fatty acids imported into mitochondria by carnitine cycle are converted through β-oxidation to acetyl-CoA.

Acetyl-CoA is considered to be a starting compound of the citric acid cycle localized in the mitochondrial matrix. In this cycle, CoA is released and the carbon acetyl skeleton is oxidized into CO_2, whereas the hydrogen atoms of substrate are carried to NAD^+ or FAD.

Subsequent electron transport from NADH or $FADH_2$ to O_2 is carried out by the respiratory chain localized in the inner mitochondrial membrane and comprised of four complexes (Saraste, 1999).

The electrons from NADH pass through the complex I and from $FADH_2$ through the complex II to the ubiquinone (UQ) and complex III transfers electrons from reduced UQ to cytochrome c (Garrett and Grisham, 1995, Saraste, 1999).

Cytochrome c is loosely associated with the inner membrane of the mitochondrion and is released by the mitochondria in case of increased permeability of mitochondrial outer membrane due to action of several cell injuring factors such as ischemia, reactive oxygen species and high Ca^{2+} (Borutaite et al., 1999, Borutaite et al., 1996, Kannan and Jain, 2000). Released to cytoplasm, initiates cytochrome c there apoptosis (Borutaite et al., 2008, Borutaite and Brown, 1998).

The final step of respiratory chain is complex IV-cytochrome oxidase that carries electrons from cytochrome c to molecular oxygen, reducing it to H_2O.

Complexes I, III and IV transports also protons from mitochondrial matrix to mitochondrial intermembrane space (IMS). The energy of achieved gradient of protons (the protonmotive force) is used for phosphorylation of ADP (generation of ATP) (Saraste, 1999).

In muscle cells, 60−80% of ATP is used directly for contraction mediated by actomyosin ATPase (Davies, 1971, Opie, 1969, Saks et al., 1998b, Stanley and Chandler, 2002), 15−25% is used for supporting the ion transport (Langer, 1974, Saks et al., 1998b), and the rest is used for maintaing signalling and anabolic reactions (Saks et al., 1998b).

The contraction cycle represents a cooperative activation of electrical and contractile processes through the excitation-contraction coupling mechanism. The contraction is triggered by the wave of electrical depolarization along the cell surface and T-tubules. The electrical changes are translated into Ca^{2+}

movements through dihydropyridine receptors (DHPR) that serve as voltage sensors (Bezanilla, 2000, Catterall, 2000, Hammes et al., 1998) and control opening of the Ca^{2+} channels in sarcoplasmic reticulum (SR) – ryanodine receptors (RyR). In cardiomyocytes, the voltage-dependent Ca^{2+} current through the DHPR Ca^{2+}-channel is sufficiently fast and large to activate the RyR in cardiac SR by a process termed Ca^{2+}-induced Ca^{2+} release (Cannell et al., 1995, Fabiato and Fabiato, 1975). In skeletal muscles, the DHPR cause activation of RYR through transmitting voltage-mediated signal to these structures that removes obstructing the pore of the channels that initiates relase of Ca^{2+} from the SR. SR represents a large intracellular Ca^{2+} store, containing this ion in amounts sufficient to fully activate the actomyosin ATPase in myofilaments (Bers, 1989, Solaro and Briggs, 1974). When [Ca^{2+}] rises in the cytosol, it binds to troponin C (TnC) and through causing conformational changes in tropomyosin relieves the sites on the actin filament to which the myosin heads of the thick filaments can cyclically bind and perform the work (Zot and Potter, 1987). Relaxation occurs when Ca^{2+} is removed from the cytosol that promotes dissociation of Ca^{2+} from TnC. In decreasing the cytosolic Ca^{2+} concentrations, the most important role belongs to the SR Ca^{2+}-pump; however, significant amounts of Ca^{2+} are also extruded from the cell by sarcolemmal (SL) Ca^{2+}-ATPase/pump and Na^+/Ca^{2+} exchanger. The SL Ca^{2+}-pump (Caroni and Carafoli, 1980) is characterized by high affinity for [Ca^{2+}] but low Ca^{2+} transport rate (Dixon and Haynes, 1989). Nevertheless, it might be important in long-term extrusion of Ca^{2+} by the cell (Bers, 1991). The Na^+/Ca^{2+} exchange system represents the main route for Ca^{2+} extrusion of the muscle cell, mainly in diastolic phase, with a capacity to remove 20−30% of Ca^{2+} from the cytosol (Reeves and Philipson, 1989, Reeves and Sutko, 1979). The Na^+/Ca^{2+} exchanger is functionally coupled to sarcolemmal Na^+/K^+-ATPase which removes entered Na^+ ions from the cytosol (Langer, 1974, Schwartz and Adams, 1980).

2. Mechanisms of cross-talk between mitochondria and ATPases in oxidative muscle cells

2.1. A change in paradigm: mitochondria and ATPases are not linked to each other by simple diffusion of adenine nucleotides

According to the classical concept, increased cytosolic [ADP] due to ATP splitting by ATPases is a main signal for cellular respiratory stimulation (Chance et al., 1985, Chance and Williams, 1956). The theory is based on following observations: 1) ADP added to isolated mitochondria activates respiration in accordance with Michaelis-Menten kinetics and with high affinity for ADP (Km=10−20 μM) (Chance and Williams, 1956); 2) experiments using saponin-skinned fibers have demonstrated that fast-twitch muscles (e.g. *m. gastrocnemius*) display high apparent affinity to ADP (Km=10−20 μM)

which is comparable to that in isolated mitochondria (Kuznetsov et al., 1996, Veksler et al., 1995); 3) data from ^{31}P-NMR studies indicate that changes in cellular respiration are associated with cytosolic ADP fuctuations in fast-twitch glycolytic muscle cells (Kushmerick et al., 1992). These findings have converged on generally accepted view that ATP produced by mitochondria diffuses to ATPases which use it as a source of energy for ion transport or contractile work. On the other hand, ADP and Pi, the products of the ATPase reaction, serve as the feedback molecules diffusing to mitochondria and stimulating there a synthesis of new ATP.

However, many of more recent data suggest that this theory can not be universally applied, that is it may be valid for fast-twitch glycolytic but not for slow-twitch oxidative muscle cells, e.g. myocardium. 1) Compared to fast-twitch muscles or isolated mitochondria the permeabilized (skinned) fibers of slow-twitch muscles exhibit much lower affinity to ADP (Km = 200–400 μM, (Kuznetsov et al., 1996, Kümmel, 1988, Liobikas et al., 2001, Saks et al., 1989, Saks et al., 1995, Seppet et al., 1991, Veksler et al., 1995). These differences can not be related to differences in muscle cell geometry (Kuznetsov et al., 1996). 2) Skinned cardiac fibers with osmotically disrupted outer mitochondrial membrane (MOM) or treated with proteolytic enzymes exhibit much lower apparent Km than their intact counterparts, whereas proteolytic treatment had no effect on Km in fast-twitch muscle fibers. The latter finding led to a proposal that in oxidative muscle cells the ADP diffusion may be restricted at the level of some intracellular structure, e.g. porin pores by some cytoplasmic, hitherto unidentified proteins (called factor 'X'), which being associated with cytoskeleton become disrupted and separated from mitochondria during their isolation (Kuznetsov et al., 1996, Saks et al., 1994, Saks et al., 1995, Saks et al., 1993). 3) It has been found that the relationship between [ADP] in the medium and respiration rate of mitochondria *in situ* in skinned fibers is highly dependent upon the source of ADP. Indeed, when the regulation of mitochondrial respiration by exogenous ATP which diffuses to ATPases and hydrolyses to ADP and Pi which in turn stimulate OXPHOS was assessed the very interesting results were obtained. The apparent Km for exogenous ATP was found to be similar to that for exogenous ADP (e.g. 300 μM for cardiac and *m. soleus* fibers); likewise, the respiration rates were similar with exogenous ATP and ADP (Seppet et al., 2001). However, despite similar respiration rates the concentrations of ADP in the medium measured chromatographically were completely different depending on the type of adenine nucleotide (ATP or ADP) added. With exogenous ADP, its steady state concentration was close to that initially added, and maximum respiration was achieved at 2 mM. In contrast, with exogenous ATP high respiration rates were observed at much lower concentration of ADP (40 μM) accumulating in the medium (Seppet et al., 2001). This phenomenon is similar to that first described by Kümmel (Kümmel, 1988) who observed high values for an apparent Km for exogenous ADP in regulation of respiration in permeabilized isolated cardiomyocytes and showed that this parameter became lower when ADP was produced in the

Ca,Mg-ATPase reaction. Thus, studies by Kümmel, 1988, Seppet 2001, and Saks 2001 have led to conclusion that endogenous ADP produced by ATPases not easily equilibrates with ADP in a bulk-water phase (which is probably the cytoplasm in the cells *in vivo*); instead, diffusion of ADP from ATPases to the cytoplasm is restricted, which explains the low [ADP] in the incubation medium. 4) In cardiac muscle, the increased energy demand with enhanced ATP production when the workload increases are not associated with changes in concentrations of cytosolic ADP (Balaban et al., 1986, Neely et al., 1972, Neely et al., 1967). Overall, the evidence collected shows that regulation of OXPHOS with ADP appears to be entirely different in oxidative and glycolytic muscle cells; in contrast to situation in glycolytic muscle cells, OXPHOS can not be upregulated by simple diffusion of ADP in oxidative muscle cells. Hence, there must exist other mechanisms linking ATPases with mitochondria.

2.2. The evidence of direct channeling of ADP

The results described above can be explained so that in oxidative muscle cells ADP produced in the intracellular ATPase reactions is most probably directly channelled to mitochondria without significant equilibration with its cytoplasmic pool in permeabilized cells (Braun et al., 2001, Saks et al., 2001, Seppet et al., 2001). This hypothesis was tested by using exogenous ADP trapping system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP), which competes with mitochondria for ADP (Fig. 1). It was assumed that the PK+PEP system added in appropriate amounts would be able to trap all ADP in the space being in equilibrium with cytoplasm, whereas ADP released into the microcompartments isolated from the cytoplasmic bulk water phase by cytoskeletal protein barriers remains unaccessible for this system. In these experiments, the concentration of PEP and activity of added PK were chosen to be 3 mM and 20 IU/ml, respectively. This PK activity, exceeding the combined rates of the ATPase and OXPHOS in oxygraphic cells by two orders of magnitude (Gellerich and Saks, 1982; Saks et al., 1984) was proved immediately rephosphorylate all ADP added to the isolated heart mitochondria and to permeabilized cardiac fibers (Seppet et al., 2001). Fig. 1 shows that without the PK+PEP system the acceptor control ratio of respiration (ACR) for saturating concentrations of ADP (2 mM), in the presence of Mg^{2+}, was about 10 (Fig. 1A), very close to that in experiments with isolated mitochondria (Seppet et al., 2001). Addition of exogenous ATP (2 mM) activated respiration about 4–5 times and the subsequent addition of ADP (2 mM) increased respiration further to the maximum level (Fig. 1B). However, as Fig. 1C shows, PK+PEP system could suppress the ATP dependent respiration not more than by 20–30%. When the PK+PEP system was added before addition of exogenous ADP, the latter was instantly converted into ATP as indicated by low ACR=4 (Fig. 1D). Thus, these experiments led to conclusion that the endogenously produced ADP is not completely released into cytoplasm (medium), but preferably available to

mitochondria, which means that ADP is directly channelled from the endogenous ATPases to mitochondria.

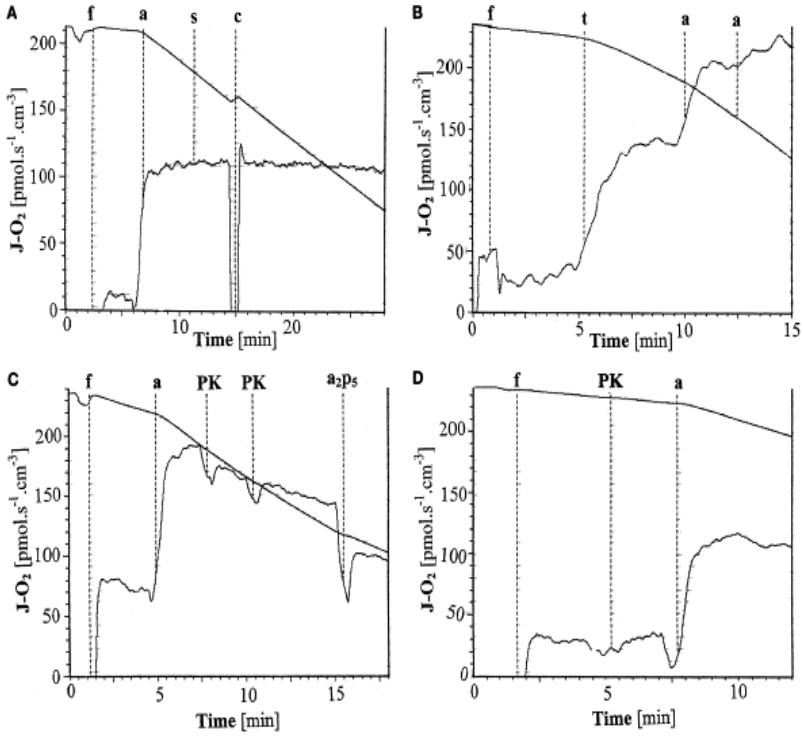

Fig. 1. **Oxygraphic recordings of the effect of the exogenous ADP trapping system of PK and PEP on the respiration of skinned cardiac fibers induced by exogenous ATP or ADP. A:** Respiratory parameters of the skinned rat cardiac fibers. Upper trace – oxygen concentration changes in time; lower trace – the first derivative of the upper trace, the rate of respiration. Additions: fibers – permeabilized fibers; ADP – 2 mM; succinate – 2 mM; cytochrome c – 8μM. The reaction rates were measured in KCl medium. The ratio of respiration rates after and before ADP addition is called ACR and is close to 10. Absence of any effect of cytochrome c addition shows perfect preservation of the outer mitochondrial membrane (see Saks et al., 1995). **B:** First ATP, 2 mM, and then ADP, twice 1 mM, in total 2 mM were added. ATP gave only about 70% of Vmax of respiration. Note that the final value of ACR after addition of ADP was close to 8. These and all further measurements were made in solution B with 4 mM MgCl2. **C:** The respiration of skinned fibers was initiated by addition of ATP, 2 mM, in the presence of PEP (5 mM) in the medium. Then PK, 20 IU, was added twice and finally AP5A (0.1 mM) was added. **D:** The medium contained 5 mM PEP and 10 IU/ml of PK. Respiration was started with addition of 2 mM ADP, but ACR 3 shows that ADP was instantly converted into ATP (see B and C) (Seppet et al., 2001, with permission from Elsevier).

As another and novel approach to demonstrate the direct channeling of ADP, the ADP flux through PK+PEP system was measured spectrophotometrically using lactate dehydrogenase (LDH) and NADH (Fig. 2). In this coupled system, the rate of oxidation of NADH is equivalent to the rate of ADP release into the medium. It can be seen that addition of MgATP to the medium activated the total ATPase (which due to the presence of Mg^{2+} and Ca^{2+} ions can be considered as a sum of the myofibrillar MgATPase and SR CaMgATPase) (Fig. 2A).

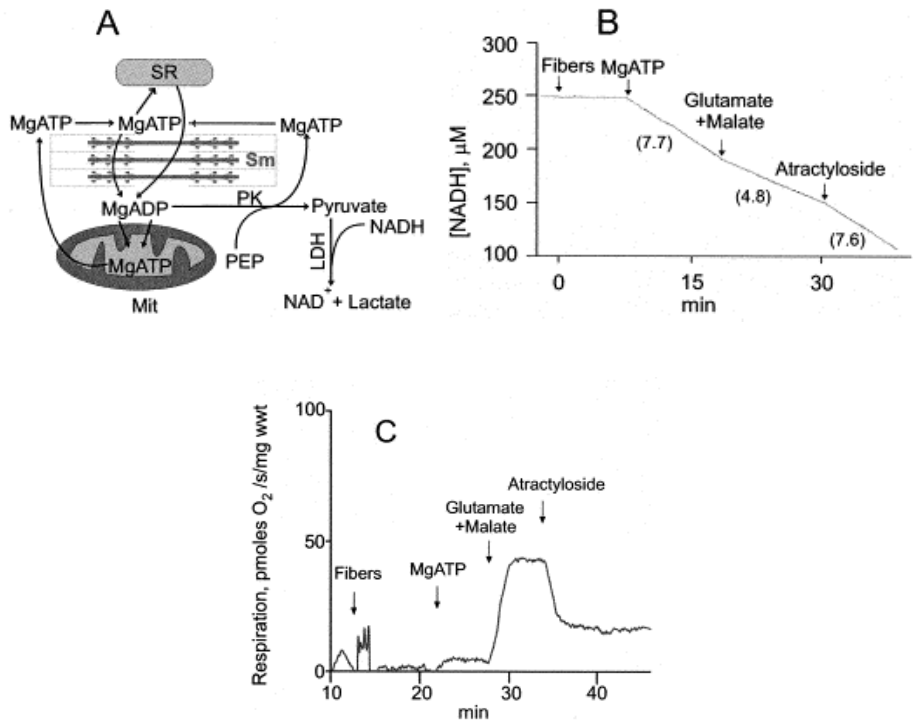

Fig. 2. Example of the analysis of rivalry between mitochondria and the external PK-PEP system for ADP generated in ATPase reactions (A) by measurements of the PK reaction (B) and respiration (C) in skinned cardiac fibers of wild-type mice. The experiments were performed at 25°C in solution B complemented with 5 mg/ml BSA, 5 mM PEP, 20 IU/ml PK, 20 IU/ml LDH, and 250 μM NADH. Further additions: fibers, 1 mM MgATP, 10 mM glutamate+2 mM malate, and 98 μM atractyloside. The numbers in parentheses indicate the rate of NADH oxidation (μM/min) in the presence of 2.6 mg/ml fibers (**B**) (Braun et al., 2001, with permission from Elsevier).

In this phase of the experiment, ADP was regenerated by the PEP-PK system as indicated by the high rate of NADH oxidation (Fig. 2B). The following addition of glutamate and malate immediately suppressed the ADP flux through the PK-PEP system, in association with rapid acceleration of respiration (Fig. 2C), because instead of the PK+PEP system the mitochondria became to phospho-rylate ADP (Fig. 2B). The following inhibition of respiration by blocking the

adenine nucleotide translocase (ANT) with atractyloside restored the ADP flux through the PK-PEP exactly to the levels registered before addition of the substrates (Fig. 2B), this also indicating involvement of mitochondria in controlling ADP flux. These results further support the hypothesis that ADP, generated by intracellular ATPases, is first available for mitochondria before its release into the medium/cytoplasm. The magnitude of direct channelling, quantitated as a difference between the fluxes prior to and after atractyloside addition, is about 50% of the maximal MgCaATPase activity (Seppet et al., 2001). Interestingly, in support of our studies, Kaasik et al (2001) have shown that in hearts of normal mice, direct mitochondrially supplied ATP was nearly as effective as ATP supplied by CK and much more effective than externally provided ATP to sustain Ca^{2+} uptake and contractile speed. Collectively, these studies show that there exists a direct ATP/ADP channelling between the mitochondria and sites of energy utilization in oxidative muscle cells.

2.3. Role of cytoskeleton in controlling ADP diffusion in oxidative muscle cells

It is well known that after selective permeabilization of the cell membrane by saponin the intracellular localization of mitochondria in relation to other cellular structures (SR, myofibrils) is well preserved, although many of the soluble proteins are washed out from the cells (Vendelin et al., 2005). The functional properties of mitochondria also remain intact (Altschuld et al., 1981, Saks et al., 2001, Saks et al., 1998a). Even after extraction of myosin with 800 mM KCl solution that produces "ghost cardiomyocytes" the intracellular localization of mitochondria does not change (Appaix et al., 2003). In contrast, mild treatment of the skinned cardiac fibers with trypsin markedly increase the affinity of mitochondrial respiration to exogenously added ADP, disorganizes mitochondrial arrangement within the cardiac cells (Fig. 3) (Saks et al., 2001, 2003) and potentiates the inhibitory influence of exogenous PK+PEP system on ATP-stimulated respiration, the latter change meaning that, after disintegration of the proteinous barriers, ADP formed in ATPase reactions becomes largely accessible for PK+PEP system (Saks et al., 2003). These experiments have led to conclusion that specific and precise intracellular organization of mitochondria arises from their interaction with cytoskeletal proteins (Saks et al., 1995, Saks et al., 1998a), which create restrictions upon intracellular diffusion of adenine nucleotides that explains high apparent Km for exogenous ADP in regulation of respiration when added to skinned cardiac fibers.

The cytoskeleton consists of different fibers classified according to their diameter as microfilaments of actin (diameter 6 nm), intermediate filaments (10 nm), such as desmin and vimentin, and microtubules (25 nm) (Rappaport et al., 1998).

Appaix et al., (2003) have shown that in permeabilized cells the mitochondria are wrapped into the microtubular network. Selective treatment of

muscle cells with trypsin results in disintegration of this network, along with disarrangement of mitochondria and decrease in Km for ADP in regulation of respiration (Saks et al., 2003).

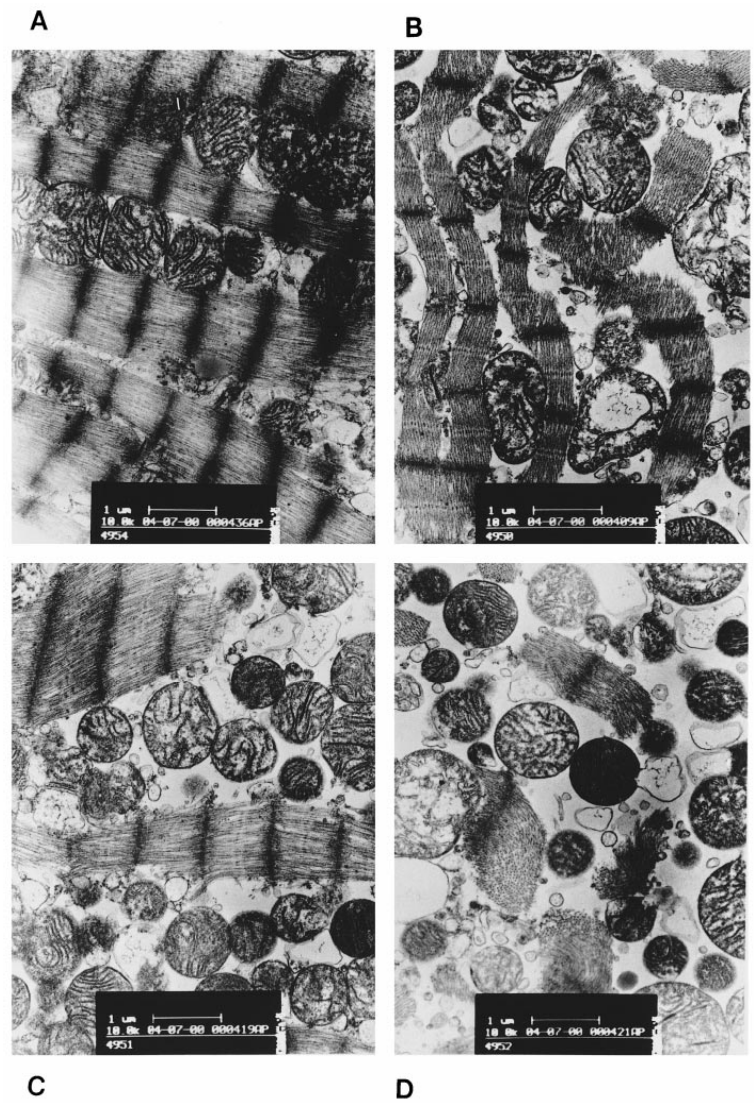

Fig. 3. Electron microscopy of skinned cardiac fibers: effects of treatment with trypsin at different concentrations on the cell structure. Fibers were treated before the fixation and observation procedure with the trypsin in the concentrations indicated below in solution B for 5 min. A, control (no treatment); B, 50 nM trypsin; C, 1 μM trypsin; D, 5 μM trypsin (Saks et al., 2001).

This observation suggests an important role of microtubules in regulation of mitochondrial function. The underlying mechanism may involve binding of the microtubules to MOM via microtubule associated proteins (Saetersdal et al., 1990). An intermediate filament – desmin connects the mitochondria with the Z-disks of the sarcomere, thereby localizing the mitochondria in a series near the A-band of the adjacent sarcomere and probably regulating the mitochondrial shape, in association with contraction-relaxation cycle of the sarcomere (Nozaki et al., 2001). Desmin also controls the mitochondrial affinity to ADP, which has been convincingly demonstrated in desmin-deficient mice. The myocardium of these animals exhibits disintegration of myofibrils and rearrangement of mitochondria, increased apparent affinity to exogenous ADP in regulation of respiration, decreased maximal rate of respiration, impaired functional coupling of mi-CK and ANT, and increased susceptibility to permeability transition pore (PTP) opening (Capetanaki, 2002, Kay et al., 1997a). It is likely that the effects of desmin on mitochondrial affinity to ADP are realized through formation and stabilization of the mitochondrial contact sites between inner and outer mitochondrial membranes, or binding to voltage-dependent anion channel (VDAC) directly or via microtubule-associated protein 2 or plectin (Leterrier et al., 1994, Reipert et al., 1999).

Recently, Kadaya et al. have studied the effects of immunoglobulin G (IgG) fraction purified from the sera of healthy persons and patients with liver disease on ADP-dependent respiration in skinned fibers of oxidative (heart and *m. soleus*) and glycolytic muscle (*m. gastrocnemius*). The results showed that IgGs from healthy persons and patients with primary biliary chirrhosis or chronic hepatitis had no effect in glycolytic muscles, but markedly inhibited respiration of mitochondria in oxidative muscles, with stronger inhibitory effect of IgGs from sick patients compared to healthy control group. Laser confocal microscopy indicated binding of IgG to the sarcomeric structures projecting at the Z-disk and M-line areas. It appears thus that the proteins of these structures might limit access of exogenous adenine nucleotides to mitochondria, thereby decreasing the rate of ADP-dependent respiration.

In conclusion, there exists ample evidence that intracellular proteins of cytoskeletal or sarcomere origin may limit diffusion of adenine nucleotides and thereby exert control over mitochondrial function. However, it is still unclear which of these proteins actually is responsible for diffusion restrictions for adenine nucleotides and whether the diffusion restrictions created are local or uniformly distributed throughout the cell's interior.

2.4. Role of creatine and adenylate kinases in intracellular energy transfer

The CK reaction ($MgADP^{-}+PCr^{2-}+H^{+}\leftrightarrow MgATP^{2-}+Cr$), discovered by Lohmann (1934), is intracellularily catalysed by different isoenzymes of CK, which have the same conserved structure of the active center (Mühlebach et al., 1994). Four

genes are responsible for encoding cytosolic M-CK, B-CK, mitochondrial sarcomeric, mi-CK_s) (sarcomeric), and mitochondrial ubiquitous (mi-CK_u) isoenzymes (Wallimann et al., 1992, Wyss et al., 1992). The cytosolic isoenzymes form dimers (MM-CK, BB-CK or MB-CK) and may be associated with cellular structures such as sarcolemma, SR and nuclei, whereas mitochondrial isoenzymes can form both octameric and dimeric structures (Wallimann et al., 1992; Wyss et al., 1992). The isoenzymes are similar in sense that they all preferably catalyze production of ATP and creatine (Wallimann et al., 1992; Wyss et al., 1992). However, their functional role may differ depending on the cell type and association to the cell structures. In fast-twitch skeletal muscles which exhibit high and fluctuating ATP consumption, the PCr/CK system functions as a reserve of energy rich phosphates, but it also transfers energy from glycolytic complexes to ATPases (Kupriyanov et al., 1980). Accordingly, most of the CK activity in these muscles is cytosolic with only 5−10% being associated with the SR and myofibrils and with the amount of mi-CK being negligible (<5%). These muscles develop a burst of intensive activity at the expenses of the energy reserves and are highly fatiguable, due to rapid loss of cellular PCr reserve. After contractile work, they require a period to recover, to regenerate cellular PCr pools through mitochondrial CK reaction (Kushmerick et al., 1992). In contrast, myocardium and *m. soleus* represent oxidative muscles with capacity of long-lasting cyclic contractile activity. In these muscle cells the CK-system serves mainly as the system of energy transfer in a form of PCr. Accordingly, these cells are rich of mitochondria (up to 40% of the cell volume), and they exhibit a high specific activity of mi-CK coupled to OXPHOS and a high relative proportion of CK is bound to other structures, but lower total CK and creatine contents compared with glycolytic muscles. For example, in cardiomyocytes at least 20% of total CK is associated with myofibrils whereas mi-CK represents 20−30% (Barbour et al., 1984, Brdiczka et al., 1994, Halestrap and Davidson, 1990, Jacobus, 1985, Jacobus and Lehninger, 1973, Muller et al., 1985, Saks et al., 1975, Ventura-Clapier et al., 1987).

In mitochondria, the mi-CK is bound to the external surface of the inner membrane by cardiolipin in a close vicinity to the ANT (Fig. 4). In octameric state the mi-CK form complexes with porin, which is a pore protein spanning the MOM, also known as VDAC (Vyssokikh and Brdiczka, 2003). Thus, porin, Mi-CK and ANT may form a three-enzyme complex in contact sites between the outer and inner mitochondrial membranes. It has been suggested that this complex is also a part of the PTP (Zoratti and Szabo, 1995). Close localization of mi-CK to ANT results in functional coupling between these enzymes. As a result, ATP generated by OXPHOS, after being transported through the mitochondrial inner membrane, is transphosphorylated to PCr with the ADP production. ADP in turn is transported back to the mitochondrial matrix by ANT (Bessman and Carpenter, 1985, Saks et al., 1994, Saks et al., 1980, Wallimann et al., 1992, Wyss et al., 1992). Such a coupling drives the CK reaction out of equilibrium towards PCr production, i.e. the reverses the

direction of CK reaction. In contrast, the cytosolic CK catalyzes the reaction of ATP-regeneration near ATPases. This process is ensured by binding of MM-CK to M-band in myofibrils and coupling to myosin ATPase (Bessman and Carpenter, 1985, Saks et al., 1978, Wallimann and Eppenberger, 1985). Similarly, in SR and sarcolemma the MM-CK is bound near the Ca^{2+} ATPase to support Ca^{2+} uptake (Korge et al., 1993, Korge and Campbell, 1994, Levitsky et al., 1978) and Na/K^{+}-pump (Grosse et al., 1980, Saks et al., 1977, Sharov et al., 1977), respectively.

Characterization of the roles of different CK isoenzymes has resulted in development of a concept of CK energy transfer and feedback signalling system in oxidative muscles (Bessman and Carpenter, 1985, Meyer et al., 1984). In this system, the PCr molecules serve as energy carriers, whereas creatine produced in CK reactions coupled to ATPases functions as a feedback metabolite as it diffuses back to mitochondria and stimulates there OXPHOS. It is likely that the CK-mediated energy transfer system operates so that release of ATP or ADP in one end of the shuttle initiates a series of near-equilibrium CK-reactions, which are transmitted through the cytosol to sites of ATP- or ADP-consumption. This mechanism seems to be a more efficient way of signalling because the diffusion distance between sites of energy production and consumption is divided into small steps, whereby relay of phosphoryl groups requires relatively small concentration gradients (Balaban et al., 1986, Dzeja et al., 1996).

Although the CK-system has been found in cardiac cells, its role may be different in atrial and ventricular myocardium. For example, although Mi-CK_S is present in rat atria, it appears not to be coupled to oxidative phosphorylation in this species (Anflous et al., 1997, Savabi and Kirsch, 1991, Vannier et al., 1996). At present, it is not clear whether such a difference is specific to rodent's heart, or it takes place also in other species including human.

AK (Kalckar, 1942) catalyses the reversible reaction: $MgADP^{2-}+ADP^{3-} \leftrightarrow MgATP^{2-}+AMP^{3-}$. In muscle cells, three AK isoforms are expressed, one of them localizing in the sarcoplasm (AK1), second in intermembrane space of mitochondria (AK2), and third (AK3) in the matrix of mitochondria (Kubo and Noda, 1974, Walker and Dow, 1982, Wilson et al., 1976). According to the current concept, the main role of AK is to participate in intracellular energy transfer, similarly to CK-system (Fig. 4). This role of AK is ensured owing to functional coupling of AK2 to ANT in the intermembrane space. As a result, mitochondrial ADP-dependent consumption of oxygen can be markedly stimulated by AMP (Dzeja et al., 1998, Gellerich, 1992). On the other hand, AK1, which is located near ATPases or ATP-dependant ion-channels, enables local rephosphorylation of ADP (Dzeja et al., 1998, Dzeja and Terzic, 1998, Terzic et al., 2000). The contribution of AK to the overall cellular ATP metabolic flux significantly increases in conditions when CK- dependent pathways become compromised, like in case of cardiac failure (Dzeja et al., 1996, Dzeja et al., 1999).

Taken together, it may be concluded that to date there exist a well documented evidence that in oxidative muscle cells the creatine and adenylate

kinases do not function as the components of ATP buffers, but represent the means of continuous and steady state energy and feedback transfer between mitochondria and ATPases.

3. Metabolic stability and the concept of intracellular energy units (ICEU)

The major issue open up to now is how do different systems of energy transfer are coordinated throughout the contraction-relaxation cycle of oxidative muscle considering 1) that intracellular diffusion of adenine nucleotides is strictly controlled in these muscle cells, 2) that muscle contraction is regulated by Ca^{2+} transients, and 3) that respiration of oxidative muscle increases linearly with workload, but without significant changes in cytoplasmic concentrations of adenine nucleotides, creatine and PCr (Balaban et al., 1986, Neely et al., 1972, Neely et al., 1967). One of the most relevant answer to this question could be that in heart muscle cells and probably in other oxidative muscle cells (*m. soleus*) mitochondria and adjacent ATPases form structural and functional complexes, termed as the intracellular energy units (ICEUs, Fig. 4) (Saks et al., 2001, Seppet et al., 2001). The borders of the ICEU, most likely made of cytoskeletal proteins (marked X) appear to isolate part of cellular adenine nucleotides and enzymes from their cytoplasmic bulk phase pool, resulting in their compartmentation into ICEUs.

Within the ICEUs energy is transferred from mitochondria to ATPases in a form of the energy-rich phosphoryl groups via specialized energy transfer systems − CK- and AK-mediated systems and direct transfer of adenine nucleotides. Cooperatively, these systems exactly match the increased energy demand with enhanced ATP energy production when the workload increases so that the cytosolic concentrations of adenine nucleotide, PCr and creatine do not change, i.e. in conditions of metabolic stability and high [ATP]/[ADP] ratio near the ATPases (Balaban et al., 1986, Neely et al., 1972, Neely et al., 1967, Nicholls and Ferguson, 2002). An important effect of such a stability is that keeping constantly high ATP/ADP local ratio ensures maximum free energy of ATP hydrolysis (Nicholls and Ferguson, 2002).

It is necessary to consider that besides the ICEU concept that explains how the processes ATP utilization and production can be metabolically linked to each other there exists a hypothesis that transient increase in cytoplasmic Ca^{2+} level that simultaneously activates the actomyosin complexes and mitochondrial enzymes, is a main factor matching the increased energy demand with enhanced ATP production („parallel activation" mechanism) (Balaban, 2002, Beutner et al., 2001, Jouaville et al., 1999, Kentish and Wrzosek, 1998, Korzeniewski, 1998, McCormack and Denton, 1990, Robb-Gaspers et al., 1998, Shimizu et al., 2002).

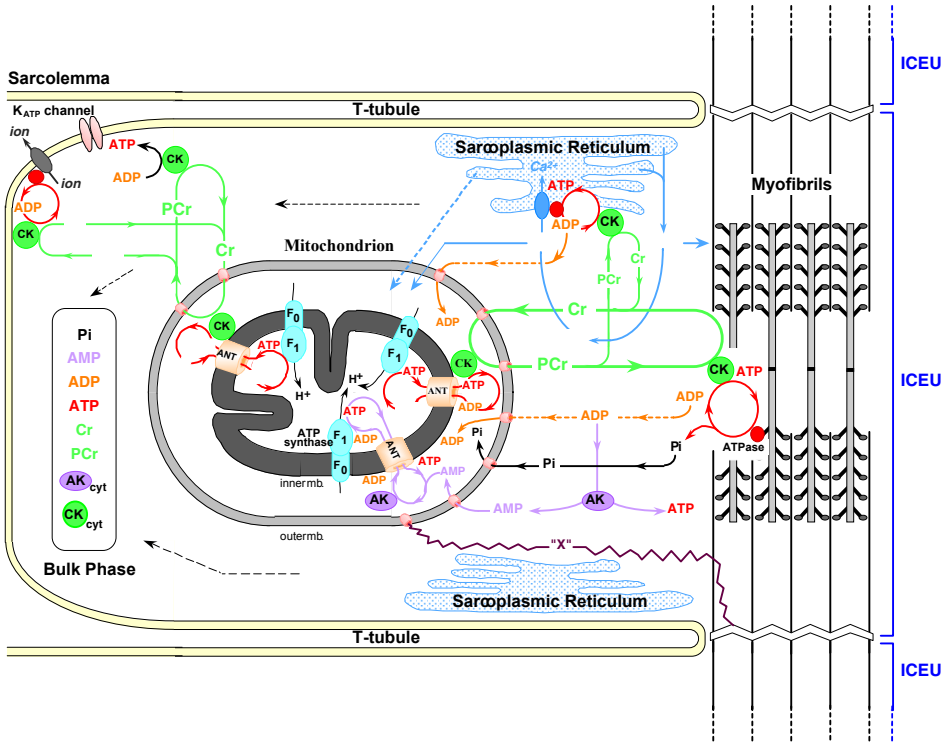

Fig. 4. Schematic presentation of functional ICEUs in the muscle cells. By interaction with cytoskeletal elements, the mitochondria and SR are precisely fixed with respect to the structure of sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Ca^{2+} is released from the SR into the space of the ICEU in the vicinity of the mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within the ICEU do not equilibrate rapidly with adenine nucleotides in the bulk-water phase. The mitochondria and ATPases of SR and myofibrils are interconnected by the creatine kinase (CK) and adenylate kinase (AK) systems, and direct channeling of adenine nucleotides. The protein factors (still unknown and marked as `X'), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP. This increases the microcompartmentation of adenine nucleotides within the ICEU and the importance of processes of vectorial ligand conduction, instead of free diffusion, as a basis of macro-osmotic processes. The ATP in the bulk-water phase may constitute a cellular metabolic reserve or serve some regulatory purposes. Adenine nucleotides within the ICEU and bulk-water phase may be connected by some more rapidly diffusing metabolites as creatine (Cr) or phosphocreatine (PCr). Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, Pi or PCr) and/or synchronized release of Ca^{2+} during the excitation-contraction coupling process (Saks et al., 2001).

Concept of „parallel activation" is based on three major lines of evidence: 1) Mitochondria can accumulate Ca^{2+} ions (Dhalla, 1969, Rossi et al., 1967), down

its electrochemical gradient (about –180 mV) through the mitochondrial inner membrane via a uniport system (Bers, 2002). 2) Mitochondrial influx of Ca^{2+} is balanced by its extrusion through coupled Ca^{2+}/Na^{+} (Crompton et al., 1977, Wingrove and Gunter, 1986) and Ca^{2+}/H^{+} exchangers (Fiskum and Lehninger, 1979, Jacobson and Duchen, 2004). By these means equilibrium of Ca^{2+} between the cytoplasmic and matrix compartments is strictly controlled. 3) Ca^{2+} stimulates the activity of several mitochondrial enzymes participating in the Krebs cycle (PDH, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) and in ATP synthesis (ATPsyntase) in vitro, this effect associating with stimulation of respiration in cardiac mitochondria (Denton et al., 1972, Denton et al., 1978, McCormack and Denton 1979, Cortassa et al., 2003, Territo et al., 2001, Territo et al., 2000). 4) It has been classically known that Ca^{2+} is a major trigger of contraction of sarcomere, by activating actomyosin cycling (Zot and Potter, 1987). From all this evidence a 'parallel activation' theory was logically derived, according to which it is Ca^{2+} which by increasing in the cytoplasm not only couples excitation to contraction and stimulates ATP hydrolysis but also activates OXPHOS, thus synchronizing the processes of utilization and production of ATP.

At present it is not clear whether and how these two concepts – the ICEU theory and „parallel activation" mechanism can be interrelated. In order to address these issues it is important to study the role of normal changes in cytoplasmic $[Ca^{2+}]$ in regulation of the different parameters of the function of ICEU.

4. Metabolic consequencies of disintegration of the ICEUs during diseases

Principally, all diseases which affect the structure of muscle cells can be associated with impairment of the ICEUs. In many cases the cytoskeletal network is altered, which means that the structural integration of ICEUs can not be properly maintained. Among numerous pathological factors, cellular hypoxia causes disturbances in interaction of mitochondria and sarcomeres, these changes eventually leading to irreversible cell injury and death (Ganote and Armstrong, 1993, Iwai et al., 1990, Vandroux et al., 2004, Nozaki et al., 2001). These underlying processes involve disruption of desmin, which would impair linking of mitochondria to Z-disks (Ganote and Armstrong, 1993, Lockard and Bloom, 1993, Reipert et al., 1999, Tokuyasu et al., 1983) and microtubule disassembly (Iwai et al., 1990, Vandroux et al., 2004).

In a course of heart failure, alterations in expression or content of desmin (Hein et al., 2000, (Li et al., 1999, (Di Somma et al., 2004)), tubulin (Heling et al., 2000), sarcoglycans and dystrophin (Yoshida et al., 2003) have been observed. These alterations may lead to impaired interaction between mitochondria and ATPases. In support of this hypothesis, it has been shown that in dystrophin-deficient MDX mice heart the apparent Km for ADP in regulation of

respiration is markedly lower than in control in permeabilized cardiac fibers, which shows altered compartmentation (Braun et al., 2001).

In parallel to cytoskeletal changes, the system of CK-mediated energy transfer is affected in conditions of various diseases. In ischemic, failing or thyrotoxic heart, reduced intracellular PCr/ATP signal ratio together with increased ADP signal (Liu et al., 2001, Neubauer et al., 1998, Neubauer et al., 1995, Ye et al., 2001), decreased myocardial content of free creatine (Buccino et al., 1967, Nascimben et al., 1996, Seppet et al., 1985, Seymour et al., 1990), downregulated expression of mi-CK (De Sousa et al., 1999, Ventura-Clapier et al., 2004, Ye et al., 2001) and dyscoupling between the mi-CK and ANT due to oxidation of mi-CK by oxygen free radicals and/or nitrosylation of its SH-groups (Kaasik et al., 1999) have been revealed. CK function is also affected in Duchenne and Becker phenotypes of muscle disease, in association with impaired OXPHOS (Dunn et al., 1993, Dupont-Versteegden et al., 1994, Glesby et al., 1988), accumulation of cytosolic ADP, muscular weakness, and atrophy (Dupont-Versteegden et al., 1994, Kemp et al., 1993, Lodi et al., 1999). Impairment of CK system may exert serious impact on intracellular energy metabolism as accumulation of ADP due to its ineffective phosphorylation represents potentially dangerous outcome, for it reduces the free energy of ATP hydrolysis (Nicholls and Ferguson, 2002) and directly inactivates the ATPases by product inhibition. Furthemore, excess ADP in the mitochondrial intermembrane space inhibits synthesis of PCr in coupled reactions of mi-CK and ANT. Decreased PCr synthesis in turn reduces the capacity of MM-CK coupled to ATPases to rephosphorylate ADP produced, thus further promoting its accumulation. Dysfunction of CK-phosphotransfer system may also promote cellular death via apoptosis, since coupling of mi-CK to ANT protects mitochondria from opening of the PTP (Dolder et al., 2003). Increasing evidence points to the importance of energy deficit (Ausma et al., 2000, Cha et al., 2003, Lin et al., 2003, Mihm et al., 2001, Tomikura et al., 2003, Tsuboi et al., 2001) in causing atrial fibrillation (AF) as well.

Fortunately, heart is capable of adaptation to impaired function of CK-phosphotransfer network. 1) Along with decreased expression of miCK and MM-CK isoenzymes the expression of BB-CK isoforms significantly increases (Nascimben et al., 1996) that is considered to improve rephosphorylation of ADP near ATPases, since BB-CK has higher affinity towards ADP than MM-CK does (Younes et al., 1985). 2) In conditions of metabolic inhibition of CK or in states of hypoxia and heart failure the role of AK in intracellular energy transfer increases, since intracellular accumulation of ADP favours utilizing of its β-phosphoryls by AK for ATP formation (Dzeja et al., 1999). However, activation of AK-phosphotransfer can not fully compensate for weakened energy transfer via CK system, as the sum of the phosphoryl transfer by these two systems lags behind the rate of ATP turnover in failed heart (Dzeja et al., 1999). It means that exhange by other means, e.g. simple diffusion or direct channeling of ADP must increase. Finally, accumulation of ADP in the cyto-plasm of cardiac cells inevitably stimulates degradation of adenine nucleotides,

also a characteristic feature of diseased heart. One of the metabolites, AMP, may exert additional influences via activation of AMP-kinases, e.g. inhibition of protein synthesis known to result from energy depletion (McLeod and Proud, 2002).

It is worth of mentioning here, however, that up to now most evidence regarding the potential pathological role of changes in structure and function of ICEUs is restricted to the data from experiments on animals. Therefore, there is a need for systematical characterization of energy metabolism and parameters of regulation of mitochondrial function in situ in human muscle cells under health and disease.

AIMS OF THE STUDY

The general aims of the study were 1) to assess the structure-function relationships in more physiological conditions than limited proteolysis, by hypercontraction in normal cardiomyocytes and in HL-1 cells, 2) to investigate whether ICEUs exist also in human oxidative muscles, and 3) to investigate alterations in energy metabolism during various diseases. More specific goals were as follows:

1. To assess the role of intracellular diffusion restrictions for adenine nucleotides in organization of energy metabolism of muscle cells by fitting the results of mathematical modelling with the real experimental data.
2. To characterize the influence of Ca^{2+}-induced hypercontraction on regulation of mitochondrial respiration and channelling of endogenous ADP in cardiac skinned fibers.
3. To characterize intracellular organization of energy metabolism in novel cultured cardiac HL-1 cell line with distinct structural properties compared to normal cardiomyocytes.
4. To characterize the CK- and AK-mediated phosphotransfer systems in human heart atria and MGM and demonstrate that the CK- and AK-phosphotransfer networks and direct channeling of adenine nucleotides are compartmentalized in these human tissues into the ICEUs.
5. To characterize alterations in energy metabolism in human atria in conditions of AF.
6. To assess the parameters of energy transfer systems in MGM in relation to osteoarthritis (OA).

METHODS

1. Animals

Adult outbred Wistar rats of either sex weighing 200–350 g or male mice (25–35 g) were used in the experiments. The animals were kept, fed and studied in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2. Patients

The investigation conforms with the principles outlined in the Declaration of Helsinki (1997) and was undertaken with written consent from the patients and agreement of the Tartu University Ethical Committee.

2.1. Patients undergoing cardiac surgery

Fifty-nine patients undergoing coronary artery bypass graft surgery, mitral or aortic valve replacement/correction and correction of atrial septal defect at the Department of Cardiovascular and Thoracic Surgery of the University of Tartu were divided into two groups. The sinus rhythm group (SR-group) (n = 44), 25 males and 19 females, having a normal sinus rhythm and the patients of the "fibrillation" group (F-group) (n = 15), eight males and seven females, associated with the persistent AF.

Compared to SR-group, the F-group patients exhibited enlarged right atrial chamber, more frequent and severe tricuspidal insufficiency, and pulmonary hypertension, all characteristic features of AF (Aime-Sempe et al., 1999, Pozzoli et al., 1998). The SR- and F-groups did not differ by age (61.6 ± 1.7 and 60.7 ± 2.4 years, respectively) and gender. Part of the atrial specimens obtained during surgical operations was rapidly frozen in liquid nitrogen and stored at −70 °C for enzyme analysis, whereas another part was permeabilized (skinned) by saponin as described (Braun et al., 2001, Saks et al., 2001, Saks et al., 1998a, Seppet et al., 2001) and used for oxygraphical and morphological studies.

2.2. Patients undergoing hip joint surgery

Sixty sedentary subjects (31 males and 29 females, 65 ± 2.4 and 66 ± 2.2 years old, respectively) participated in this study. The patients were divided into three groups. The control group (n = 15, 10/5 male/female ratio, age 68 ± 5.2) comprised the patients undergoing surgical correction of traumatic hip fracture. The two other groups included the patients with unilateral or bilateral hip

replacement for OA of radiographic grade 3 (n = 11, 8/3, age 66 ± 4.1) and grade 4 (n = 34, 13/21, age 65 ± 1.2) estimated according to Kellgren and Lawrence (Kellgren and Lawrence, 1957). The muscle specimens (50−100 mg) were taken during surgery from the middle portion of the MGM. A part of each specimen was rapidly frozen in liquid nitrogen and stored at –70ºC for enzyme and myosin heavy chain (MHC) analyses, whereas the rest was permeabilized (skinned) by saponin (Braun et al., 2001, Seppet et al., 2001) and used for oxygraphical studies.

3. Cardiomyocyte isolation and cell culturing

Adult cardiomyocytes were isolated from rat heart by perfusion with a collagenase-containing medium and permeabilized by incubating with saponin as described previously (Kay et al., 1997a; Saks et al., 1991; Saks et al., 1998a). The B HL-1 cell line was developed from atrial cardiac myocytes from transgenic mice grown in a specific medium (Claycomb et al., 1998) and transferred into the NB HL-1 cell line as described previously (Pelloux et al., 2006).

4. Preparation of ghost cells and fibers

The ghost cells and fibers, deficient in myosin were prepared by application of high KCl-technique for solubilization of myosin as previously described (Kay et al., 1997a, Saks et al., 1993).

5. Isolation of mitochondria

Mitochondria were isolated from rat hearts using differential centrifugation technique as described previously (Saks et al., 1975).

6. Respirometric investigations

The function of the respiratory chain in skinned muscle fibers or cardiac cells was assessed by polarographic method (Oroboros, Paar KG, Austria or Rank Brothers Ltd., England) as respiration rates (V or VO_2) in solution B containing (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3, and 5 mg/mL fatty acid free bovine serum albumin (BSA), glutamate or pyruvate 10 and malate 2, pH 7.1 at 25ºC, in conditions of subsequent addition of 2 mM ADP to register the NADH-linked ADP-dependent respiration rate in the presence of glutamate (V_{Glut}) or pyruvate (V_{Pyr}), 10 μM rotenone to inhibit the complex I, 10 mM succinate to activate

$FADH_2$-linked ADP-dependent respiration (V_{Succ}), 0.1 mM atractyloside to monitor the respiratory control by adenine nucleotide translocase (ANT), 10 μM antimycin A to inhibit the electron flow from complex II to cytochrome c, 0.5 mM TMPD and 2 mM ascorbate to activate cytochrome oxidase (V_{COX}), and 8 μM cytochrome c to test the intactness of MOM (Saks et al., 1998a). The antimycin-sensitive respiration in the presence of atractyloside was considered to represent the proton leak. The COX activity was measured as the NaN_3-sensitive portion of the TMPD-dependent VO_2.

The coupling between OXPHOS and mi-CK was estimated in solution B supplemented with 10 mM glutamate or pyruvate and 2 mM malate by two means. First, VO_2 versus [ADP] relationships were examined in the presence and absence of 20 mM creatine and the interaction between mi-CK and ANT was expressed as [Km_{ADP}(−Cr)/Km_{ADP}(+Cr)] (creatine index). Second, coupling of mitochondrial AK (mi-AK, i.e. AK2 isoform) to ANT was quantified after addition by 2 mM of AMP in the presence of 50 μM ATP as the AK index (I_{AK}) calculated from equation $I_{AK} = [(V_{AMP} - V_{ATP})/V_{ATP}]$, where V_{AMP} and V_{ATP} are the respiration rates with AMP and ATP, respectively. After AP_5A inhibition of AK 20 mM creatine was added to couple the mitochondrial CK (mi-CK) to ANT. The efficiency of coupling was expressed as the CK index (I_{CK}): $I_{CK} = [(V_{Cr} - V_{AP5A})/V_{AP5A}]$. Then 2 mM ADP was added to maximally stimulate the OXPHOS (V_{ADP}). In the same protocol, the intactness of the mitochondrial inner membrane or MOM was controlled by addition of 0.1mM atractyloside or excess cytochrome c (8 μM), respectively (Saks et al., 1998a). The maximal capacity of the respiratory chain was estimated as the VO_2 with 2 μM FCCP (V_{FCCP}). To demonstrate that coupling of mitochondrial kinases results in local ADP production near ANT, approximately 3–4 mg of fibers were incubated in solution B at 25 ºC in the presence of 10 mM glutamate, 2 mM malate and 5 mM PEP and changes in respiration were assessed after subsequent additions of 50 μM or 2 mM ATP, 20 IU/mL PK, 20 mM Cr or 2 mM AMP, and 2 mM ATP. The experiments were finished by addition of 0.1 mM ATR to monitor the respiratory control by endogenously produced ADP and intactness of the inner mitochondrial membrane (Saks et al., 1998a).

To analyze the mitochondrial function in B HL-1 and NB HL-1 cells, the cells were detached by trypsinization, trice washed and centrifuged for 5 min at 1000 rpm with phosphate-buffered saline (PBS) at 4 °C. Subsequently, the sediment was resuspended in in the modified Mitomed solution (Pelloux et al., 2006) of the following composition (in mM): sucrose 110, K-lactobionate 60, CaK_2EGTA 0.138, K_2EGTA 0.362, $MgCl_2$ 3, dithiothreitol 0.5, taurine 20, KH_2PO_4 3, K-HEPES 20, pH 7.1, with 1 mg/ml BSA. The free [Ca^{2+}] in this solution was 0.2 μM as detected fluorimetrically (FlexStation II, Molecular Devices Corporation, USA) by using the Ca^{2+} calibration buffer kit and indicator fura 4F (Molecular Probes Europe BV, Leiden, Netherlands). Where required, ATP or ADP were added together with $MgCl_2$ (0.8 mol/mol for ATP and 0.6 mol/mol for ADP) to keep free Mg^{2+} constant in the medium.

The HL-1 cells or for control permeabilized cardiac fibers/cardiomyocytes were incubated in an oxygraph chamber. For permeabilization of the cell membrane, 50 μg/ml saponin was added and the cells were incubated during the 15 min. Then the analysis of the function of the respiratory chain was started by adding 10 mM glutamate and 2 mM malate. After the registration of basal respiration rate in non-phosphorylating conditions (V_0), 2 mM MgADP was added to monitor the maximum rate of NADH-linked state 3 respiration (V_{Glut}), followed by successive additions of 10 μM rotenone to inhibit the complex I, 10 mM succinate to activate $FADH_2$-linked state 3 respiration (V_{Succ}), 0.1 mM atractyloside to assess respiratory control by blocking ANT (V_{Atr}), 10 μM antimycin A to inhibit the electron flow from complex II to cytochrome c (V_{Ant}), 0.5 mM TMPD with 2 mM ascorbate to activate cytochrome oxidase (COX), 8 μM cytochrome c to monitor the intactness of the MOM, and 5 mM NaN_3 to quantify COX activity as a NaN_3-sensitive portion of respiration (V_{COX}). Proton leak was calculated as a difference between the respiration rates in the presence of atractyloside and antimycin A (V_{Atr}–V_{Ant}).

Then the analysis of the coupling between OXPHOS and mitochondrial kinases in HL-1 cells was performed essentially similarly to that in skinned muscle fibers (above) except that the HL-1 cells were incubated for 15 min in Mitomed medium at 25 ºC in the presence of 50 μg/ml saponin to permeabilize the cells in the oxygraph chamber, and 10 mM succinate and 10 μM rotenone to measure the complex II dependent respiration. After stabilization of the respiration at its basal levels (V_0), the following additions were made: 0.1 mM MgATP, 2 mM AMP, 0.2 mM AP_5A, and 20 mM creatine. The experiment was continued by adding 2 mM MgADP to gain the maximum rate of OXPHOS, followed by the addition of 0.1 mM atractyloside to monitor the respiratory control via ANT. Then 200 nM FCCP was added to register the maximum uncoupled respiration and 8 μM cytochrome c was added to assess the intactness of the MOM.

In the second protocol, after registration of V_0 in the presence of 5 mM PEP, 2 mM MgATP, 20 IU/ml PK and 20 mM creatine were successively added. The same protocol was applied to estimate the coupling between mitochondrial AK2 (mi-AK2) and OXPHOS, except that 20 mM creatine was replaced by 2 mM AMP.

To assess the role of HK in stimulating OXPHOS the cells were permeabilized as described above and incubated in the presence of 0.1 μM MgATP. Then 10 mM glucose was added followed by additions of 2 mM MgADP and 0.1 mM atractyloside. In separate experiments, the role of HK bound to mitochondria was analysed by the same protocol but after the washout of the cytosolic enzymes as following: The cells were incubated in shaking conditions in 3 ml Mitomed solution with 50 μg/ml saponin for 10 min at room temperature and centrifuged at 1000 rpm for 5 min. Then the supernatant was removed, and the pellet was resuspended in Mitomed and centrifuged 1000 rpm

5 min, this washing step was repeated twice more, and the final pellet was resuspended in Mitomed and used for experiments as a stock solution.

7. Determination of the direct channeling of adenine nucleotides between mitochondria and ATPases

The suspension of HL-1 cells or fibers was incubated in the spectrophotometric (Perkin-Elmer Lambda 900) cuvette containing Mitomed or B solution supplemented with 5 mM PEP, 20 IU/ml PK, 20 IU/ml LDH and 0.24 mM NADH at 25ºC. The medium was continuously mixed with a magnetic stirrer operated by the Variomag Telemodul (H+P Labortechnic, Germany). To permeabilize the HL-1 cells, 50 μg/ml saponin was added and the cells were incubated for 15 min before the following successive additions were made (fibers and adult cardiomyocytes were permeabilized before): 2 mM MgATP, 10 mM glutamate and 2 mM malate (or 10 mM succinate with 10 μM rotenone), and 0.1 mM atractyloside. The extent of mitochondrial rephosphorylation of ADP produced in the CaMgATPase reactions was quantified as the flux through the PEP-PK system registered by the changes in [NADH] at 340 nm decreased after switching on the OXPHOS by adding respiratory substrates. (Seppet et al., 2001). A coupling of cytosolic CK isoforms to CaMgATPases was assessed from the decrease of the ADP flux through the PK-PEP system caused by 20 mM phosphocreatine (PCr) added after stabilization of the ADP flux in the presence of atractyloside.

8. Electron microscopy

After having registered the respiration rates, the skinned fibres were removed from the oxygraph, slightly blotted by filter paper, fixed in 0.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in the same buffer. After dehydration with ethanol and acetone, the specimens were embedded in Epon 812. The sections were stained with uranyl acetate followed by lead citrate, and examined using a Tecnai 10 electron microscope (FEI Company, Netherlands) at 100 kV. For ultra structure investigations, the longitudinally orientated cardiomyocytes were selected and, from five to seven separate images corresponding to the same experimental conditions, the lengths of 92 to 390 sarcomeres were measured.

9. Confocal imaging of mitochondria in living cells

To detect the mitochondrial functional state at the level of the single mitochondrion, images were acquired and analyzed by fluorescent confocal micros-

copy. For mitochondrial imaging (localization) studies, cells were loaded with MitoTracker Green (0.2 uM) or MitoTracker Deep Red (85 nM) (Molecular Probes, USA). The cells were placed in LabTek chambered coverglass (Nalge Nunc, USA) and incubated for at least 2 h at 4°C for cardiomyocytes and 15 min to 2 h at 36°C for HL-1 cells before analysis (chamber volume 0.3 mL; $10x10^3$ to $2OxlO^3$ cells per chamber). The digital images of MitoTracker Green fluorescence were acquired with an inverted confocal microscope (Leica DM IRE2, Leica Microsystems, Germany) with a 63 x magnfication water immersion lens. The MitoTracker Green fluorescence was excited with the 488 nm line of an argon laser, using 510 nm to 550 nm for emission. Tetramethylrhodamine methylester fluorescence was measured using 543 nm for excitation (helium-neon laser) and greater than 580 nm for emission.

10. Determination of the activities of kinases

Rat ventricular myocardial or HL-1 cells were homogenized by sonication (Bandelin Sonopuls HD 2200, probe MS 72) in ice-cold PBS, muscle specimens by an Ultra-Turrax homogenizer as described before (Braun et al., 2001). The homogenates were incubated in a spectrophotometric cuvette in stirring conditions in the Mitomed medium supplemented with NADH 0.24 mM, PEP 0.8 mM, 6 IU/ml PK, and 3 IU/ml LDH (pH 7.1, 25 °C). After registration of basal CaMgATPase in the presence of 1 mM MgATP, 1.3 mM AMP was added to determine the AK activity from the changes in NADH oxidation rates at 340 nm. For CK total activity measurements the Mitomed medium was supplemented with 2 mM MgADP, 6 mM glucose, 0.6 mM NADP, 0.5 mM AP_5A, 2 IU/ml hexokinase (HK), and 2 IU/ml glucose-6-phosphate dehydrogenase (G6PDH) at 25 ºC. Then the specimen was added, and after stabilizing the optical density, the reaction was started by addition of 20 mM PCr and the rate of NADPH formation was registered. The HK activity was measured in Mitomed medium in the presence of 2 mM MgATP, 0.6 mM NADP and 2 IU/ml G6PDH, and the rate of NADPH formation was monitored spectrophotometrically (Perkin-Elmer Lambda 900) after addition of 10 mM glucose at 340 nm, 25 °C. To measure the mitochondria-bound HK activity in HL-1 cells, the cells were permeabilized with saponin (50 μg/ml, 10 min at room temperature) in the Mitomed solution as described above before assessment.

11. Determination of isoform profile of kinases

The HL-1 cells frozen at − 80 ºC were thawed at 0 ºC and homogenized in the medium containing (in mM): EGTA 1, dithiothreitol 1, $MgCl_2$ HEPES 2. 5, and 1% Triton X-100 (1:20 w/v), pH 8.7, by ultrasound on ice during 15 s followed by a 1 min period of keeping the probe on ice. Then the same cycles were

repeated thrice, and the homogenates were left on ice for 1 h for complete extraction of the CK. The CK isoform profile was assayed as follows. Samples of 4 μl (20–40 μg protein, 0.002–0.004 IU CK) of homogenates were applied to a 1% agarose gel and subjected to electrophoresis for 1h at 180 V in Tris/barbital buffer (50 mM, pH 8.9). To reveal the CK isoenzyme activities the gel was incubated with a staining solution-soaked paper for 30 min at 30ºC, and the fluorescence of the produced NADPH was visualized under UV light and analyzed with Fluor-S Multi-imager (Bio-Rad, USA). The staining solution contained (in mM) MES 22 (pH 6.8), magnesium acetate 50, glucose 70, N-acetyl cysteine 120, PCr 130, ADP 9, NADP 10, AMP 18, AP_5A 0.75, supplemented with 18 U/ml HK, and 6 U/ml G6PDH. Electrophoresis of HK was performed in 1% agarose gel in Tris/barbital buffer (50 mM, pH 8.5, 180 V) at 4°C.

12. Determination of myosin heavy chain isoform profile

MHC isoform profile was assayed by 7.2% SDS-polyacrylamide gel electrophoresis (Schiaffino and Reggiani, 1994, Smerdu et al., 1994).

13. SDS-polyacrylamide gel electrophoresis and immunoblotting

Fifty micrograms of total protein in homogenates was separated by standard 12% SDS-polyacrylamide gel electrophoresis and electrotransferred by semidry blotting (Hoefel Pharmacia Biotech Inc. San Fransisco CA, USA) on a nitrocellulose membrane (Shleicher & Schüell, Dassel, Germany). The membranes were blocked with 4% fat-free milk powder in T-TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) overnight at 4 °C, incubated for 15 min at room temperature and washed for 4 x 5 minutes with T-TBS. Then the membranes were incubated for 1h with ubiquitous mitochondrial CK rabbit immune sera (1:2000 dilution in a blocking buffer) or with affinity-purifed chicken anti-B-CK IgY (1:500 dilution in a blocking buffer) at room temperature (Schlattner et al., 2002). For detecting AKs, the membranes were incubated for 1h at room temperature with rabbit polyclonal antibodies against AK1 (H-90, Santa Cruz Biotechnology, Inc., USA) or AK2 (H-65, Santa Cruz Biotechnology, Inc., USA) (dilution 1:500 in a blocking buffer), washed for 4 x 5 min in T-TBS and incubated for 1 h with the peroxidase-coupled secondary antibody, either goat anti-rabbit IgG (Nordic, Lausanne, Switzerland) (1:1000 dilution in a blocking buffer) or rabbit anti-chicken IgY (Jackson Immuno-Research, West Grove, PA) (1:3000 dilution in a blocking buffer) and finally washed 4 x 5 min with T-TBS. The blots were developed with the enhanced chemiluminescence substrate (Amersham, Buckinghamshire, UK) and exposed to an X-ray film.

14. RNA isolation

Total RNA from $1 \cdot 10^6 - 1 \cdot 10^7$ frozen HL1 cells suspended in PBS was isolated using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The integrity of RNA was verified by 0.8% agarose gel electrophoresis. Ribosomal RNA bands 28S and 18S were visualized by ethidium bromide staining. Quantification of the nucleic acid was carried out spectrophotometrically (Lambda 900, Perkin-Elmer) at 260 nm, and the purity of RNA preparation was checked by assessing the *A*260/*A*280 ratio. Thereafter the gel was trans-illuminated with UV light and photographed by Syngene Gel Documentation System with a Syngene Software GeneSnap (Syngene, UK).

15. Reverse transcriptase reaction

For reverse transcription (RT), total RNA (approximately 500 ng) obtained from HL-1 cells was processed for single-stranded cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Karsruhe, Germany) and oligo dT (Proligo France SAS, Paris, France). All RT reactions were done in a PCR thermocycler GeneAmp® PCR System 2400 (Perkin Elmer/Applied Biosystems). The resulting cDNA was used for real-time RT-PCR performed by an ABI PRISM 7000 Sequence Detection System (Perkin Elmer/Applied Biosystems) using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). The samples in the reaction mixture, supplied with QuantiTect SYBR Green PCR Kit as well as no template controls, were loaded in a total volume of 31 μl per reaction. Thereafter gene specific primers were added into the reaction mix in the concentration of 0.3 μM. The oligonucleotide primers (Proligo France SAS, Paris, France) used for real-time PCR analysis are shown in Table 1. An identical PCR cycle profile was used for all genes. The amplification started by heat activation of HotStarTaq® DNA polymerase at 95 °C for 15 min. The following 35 cycles of PCR consisted of denaturation step for 15 s at 94°C, primer annealing step for 30 s at 59 °C and for 30 s extension phase at 72°C. The expression data were analyzed by using a comparative critical threshold method, where the amount of the gene of interest was normalized to the housekeeping gene (hypoxanthine ribosyl transferase (HPRT1) and expressed relative to the mean values of calibrator samples using the following equation (Livak and Schmittgen, 2001):

$$\text{Fold induction} = 2^{-\Delta\Delta CT} \quad (1),$$

where ΔΔCT equals to the threshold cycle difference of the gene of interest relative to reference gene HPRT1 in an unknown sample minus the threshold cycle difference of the unknown gene relative to reference gene HPRT1 in calibrator sample (adult rodent heart). The amplified cDNAs were separated in a

1.7 % agarose gel to verify amplicons by size using DNA size marker (100 bp Generuler, Fermentas, Lithuania).

16. Mathematical modeling

The mathematical models were designed to study two possible mechanisms by which functional coupling between Ca,MgATPases and mitochondria could be formed in cardiac skinned fibers. One of the mechanism assumes that diffusion within the fibers is limited uniformly by distributed diffusion restriction (Dist ICEU model), whereas another assumes that diffusion restriction is localized close to mitochondria (small ICEU model). To differentiate between these two mechanisms the spatially inhomogeneous reaction-diffusion model of energy transfer (Saks et al., 2003) was applied by considering the reactions in four compartments of cardiac cells: the myofibril together with the cytosol, the vicinity of mitochondria (VIM), the mitochondrial intermembrane space (IMS), and the mitochondrial inner membrane-matrix space. The parameters describing diffusion between compartments were F^{D} and F^{OM} for DistICEU model, and F^{IST} and F^{OM} for SmallICEU model (Paper I).

17. Reagents

The reagents were purchased from Sigma (USA), Serva (Germany), Roche (France), Boeringer (Germany) and Fluka (Switzerland).

18. Statistical analysis

Statistical analysis of data was performed by one-way ANOVA with Newman-Keuls, Bonferroni or Dunnett's post test, by Mann-Whitney test and Student's *t* test. The means ± SEM or SD are presented where indicated.

RESULTS AND DISCUSSION

1. Evidence for intracellular compartmentation of energy metabolism and the underlying mechanisms (Papers I, II, III, IV, V, VI)

1.1. Study of the structure-function relationships in conditions of different experimental models

If the ICEUs indeed play a central role in the organization of energy metabolism in cardiac cells, alterations in their structure and function should interfere with the regulation of processes of energy production and utilization, and through these processes, with cardiac contractile activity. As described above, modulation of the ICEU structure with trypsin is one of approaches to test the validity of that hypothesis. Indeed, the increased apparent affinity of mitochondria to exogenous ADP after disorganization of cellular structure by trypsin can be taken to indicate that the trypsin treatment resulted in a loss of diffusion barriers for ADP at the borders of the ICEU. However, treatment of cardiac cells with trypsin can introduce some artifacts due to non-specific and unpredictable influences on various cellular structures, including mitochondrial membranes. To overcome these problems, we have assessed the structure-function relationships in more physiological context, by applying two different cellular models: (i) hypercontraction caused by excess Ca^{2+} in normal cardiomyocytes and (ii) HL-1 cells, in which mitochondria are organized very differently compared to adult cardiomyocytes (Anmann et al., 2006).

1.1.1. Ca^{2+}-dependent contraction of cardiac muscle is associated with altered kinetics of regulation of mitochondrial function (Papers III, V)

Figs. 5 and 8 demonstrate a regular, crystal-like (Vendelin et al., 2005) arrangement of mitochondria in adult cardiomyocytes, due to precisely fixed mitochondrial positions adjacent to neighbouring sarcomeresas shown also in earler studies (Nozaki et al., 2001). The proteins responsible for exact interaction between sarcomeres and mitochondria are yet to be defined; however, the desmin filaments connecting mitochondria to Z-disks of sarcomeres may be of major importance (Nozaki et al., 2001). Furthermore, the findings that changes in sarcomere and mitochondrial lengths, caused by different means, occur in parallel manner (Nozaki et al., 2001) and that controlled swelling of mitochondria is associated with increased developed tension due to changes in physical forces in skinned cardiac fibers (Kaasik et al., 2004) suggest that structural junctions between mitochondria and sarcomeres are strong enough to transmit forces from one to another.

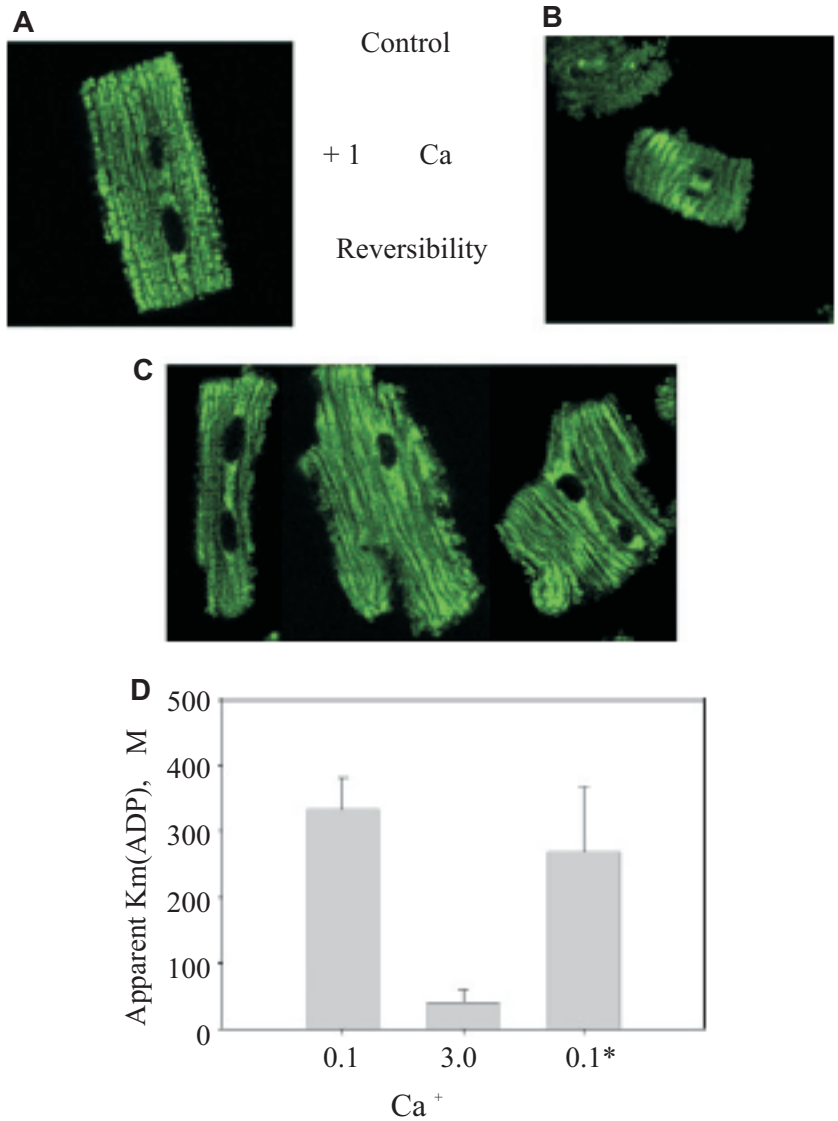

Fig. 5. Reversibility of the calcium-induced contraction of permeabilized cardiomyocytes. (**A**) Cells were incubated and mitochondrial flavoproteins were imaged at 0.1 μM free calcium. (**B**) The hypercontraction shown was induced by increasing the free calcium concentration to 1.0 μM. (**C**) Cardiomyocytes were then transferred back into solution B that contained 0.1 μM calcium. (**D**) **Reversibility of the effects of calcium-induced contraction of cardiomyocytes on the kinetics of regulation of mitochondrial respiration by exogenous ADP**. The kinetics of respiratory regulation was measured in solution B containing the respiratory substrates and 3 μM free calcium, then fibers were washed twice (7 min each wash) in solution B containing 0.1 μM calcium, and the kinetics were measured again in the presence of 0.1 μM calcium (return to this calcium concentration is shown by 0.1*). The average data for three separate experiments (±SD) are shown. Paper III.

To monitor as to whether the mechanical interactions between mitochondria and sarcomeres are also associated with changes in regulation of OXPHOS, we studied the kinetics of regulation of respiration in skinned cardiac fibers and cells in conditions of sarcomere shortening caused by increasing concentrations of cytosolic free Ca^{2+}. In the presence of ATP, increasing of free [Ca^{2+}] up to 1−4 μM induced a strong contraction (hypercontraction) of permeabilized cardiomyocytes or fibers with significant alterations in mitochondrial arrangement (Fig. 5). In parallel, the apparent Km for exogenous ADP or ATP in regulation of respiration drastically decreased, with only modest increase in Vmax of respiration (by 40%) with optimum of [Ca^{2+}] at 0.4 μM and subsequent decrease at higher [Ca^{2+}] (Paper III). All these changes were reversible after decreasing the free [Ca^{2+}] to diastolic levels (Fig. 5).

Interestingly, excess Ca^{2+} exerted no effect on contractile (Fig. 6) and mitochondrial functions (Paper III) in "ghost" cells or fibers, from which the myosin ATPase was extracted. Similar dependence of mitochondrial function on presence of contractile system has been recently observed in experiments, which showed that in contrast to intact skinned muscle fibers, where caffeine stimulated both respiration and actomyosin ATPase activity by causing Ca^{2+} efflux from SR, it exerted no effect on respiration in fibers without myosin. Thus, a conclusion was made that the primary role for Ca^{2+} ions in muscle cells is to activate contractile system, which secondarily stimulates respiration via augmented production of ADP (Khuchua et al., 1994).

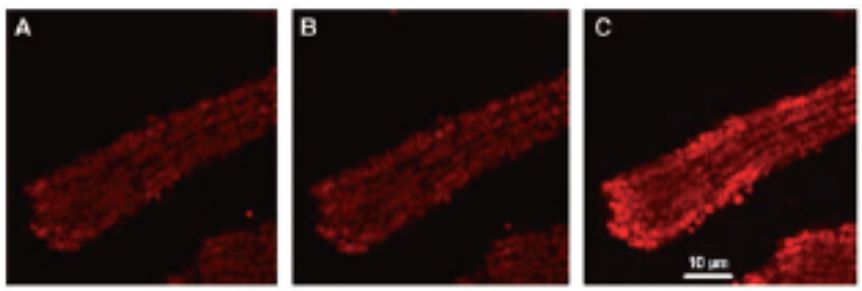

Fig. 6. Absence of contraction in 'ghost' cardiomyocytes after the addition up to 3 μM free calcium in the presence of ATP (1 mM) and glutamate (5 mM). (A) Control 'ghost' cardiomyocytes preloaded with 5 μM Rhod-2. (B) Cardiomyocytes after the addition of 1 μM free calcium. (C) Cardiomyocytes after the addition of 3 μM free calcium. A significant increase in the fluorescence intensity of Rhod-2 clearly shows an elevated calcium concentration in the mitochondrial matrix of 'ghost' cardiomyocytes, in particular after the addition of 3 μM free calcium. Paper III.

Our data clearly show that hypercontraction (marked shortening of sarcomeres at a free Ca^{2+} concentration of 2 μM (Fig. 7)) significantly decreased the mitochondrial role of providing ATP for contraction as in contracted muscle the

direct transfer of adenine nucleotides was diminished, and, respectively, increased proportion of ADP became available for phosphorylation by PK+PEP system (Paper III). A likely explanation for that phenomenon is that strong contraction caused such structural alterations in the diffusion barriers within the ICEUs, which allowed ADP to escape these units thereby limiting the extent of mitochondrial phosphorylation of ADP. At the same time, decreased Km for ADP in regulation of respiration (Paper III) indicates that exogenous ADP became easier available for mitochondria. Thus, the ICEUs' concept offers a novel mechanism for controlling interaction of ATPases and mitochondria within the contraction-relaxation cycle – via contraction-related alterations in localized restrictions of the intracellular diffusion of adenine nucleotides. The underlying mechanisms may comprise the contraction-dependent opening of the VDAC pores to adenine nucleotides and/or disintegration of the border-barriers of the ICEUs leading to decreased diffusion restriction for adenine nucleotides (see also Chapter 1.1.3).

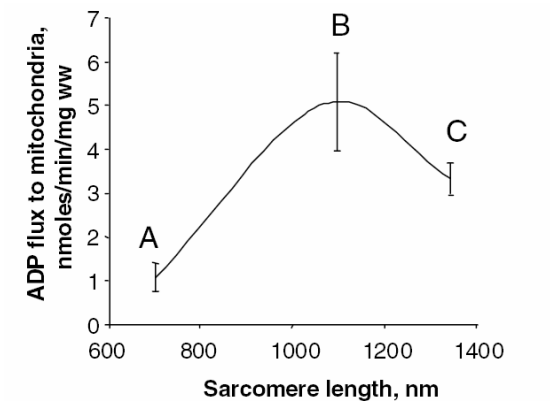

Fig. 7. Relationships between the mitochondrially phosphorylated ADP flux (means ± SEM) and mean sarcomere length altered by changing the free Ca^{2+} concentration (2 μM at point A, 0.1 μM at point B and 0 μM at point C) in permeabilized rat cardiac cells incubated in solution B, with 20 mM ATP and the PK+ PEP system (20 U/mL PK and 5 mM PEP), and in the presence of respiratory substrates (10 mM glutamate and 2 mM malate). ww – wet weigth. Paper V.

1.1.2. HL-1 cells as a native model of altered structure-function relationships in regulation of energy metabolism (Papers V and VI)

The HL-1 cells, while maintaining a differentiated cardiac phenotype through indefinite passages in culture, can continuously proliferate and spontaneously contract (beating (B) HL-1 cells) (Claycomb et al., 1998, White et al., 2004). Ultrastructural studies show that compared with adult rat cardiomyocytes

(Fig. 8A and B), characterized by perfect intracellular arrangement of mitochondria in adult cardiomyocytes, the HL-1 cells exhibit very different structural organization (Fig. 8C and D), as mitochondria in these cells are chaotically arranged and form dynamically changing filaments. Whereas the B HL-1 cells possess some residual sarcomeres (Claycomb et al., 1998, White et al., 2004), the NB HL-1 cells are devoid of these structures. When the regulation of mitochondrial respiration was studied in permeabilized HL-1 cells it was found that the mitochondria exhibited very low apparent Km for exogenous ADP in stimulation of respiration as compared to that in adult cardiomyocytes (Anmann et al., 2006)

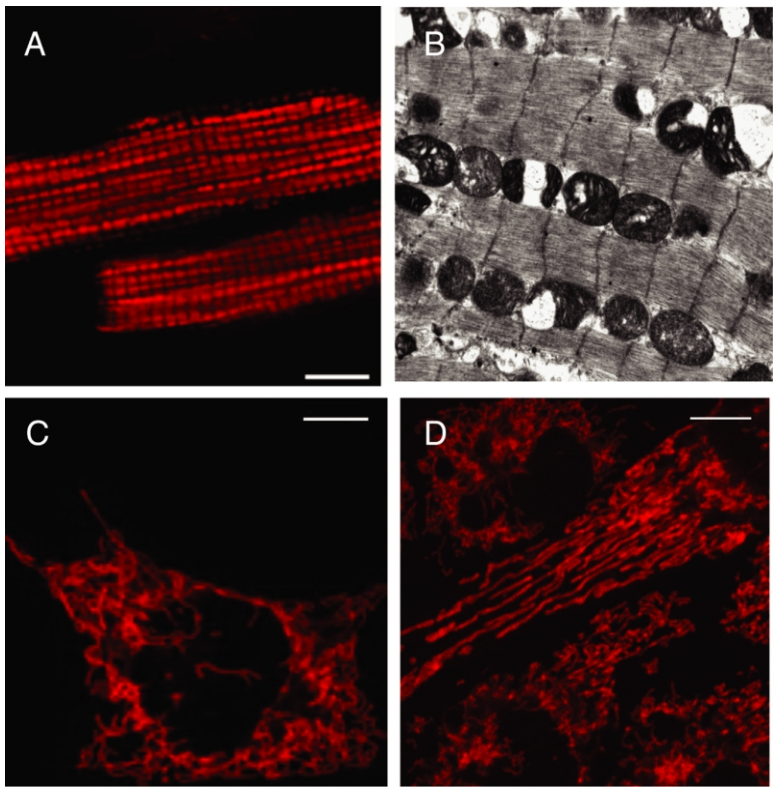

Fig. 8. Confocal (A, C and D) and electron microscopical (B) visualization of mitochondria in adult rat cardiomyocytes (A and B), and cultured nonbeating HL-1 cells (C) and beating HL-1 cells (D). A Mitochondria were visualized using MitoTracker Red CMXRos (Molecular Probes, USA) (0.1 μM) in the presence of 5 mM glutamate and 2 mM malate. **B** Mitochondrial arrangement in permeabilized rat cardio-myocyte within the skinned fiber preparation in solution B. **C** Mitochondrial arrangement and dynamics in non-beating HL-1 cells in die presence of MitoTracker Deep Red in Tyrode's medium. **D** Mitochondrial arrangement and dynamics in normally cultured beating HL-1 cells visualized using MitoTracker Deep Red. Bars represent a distance of 10 μm. Paper V.

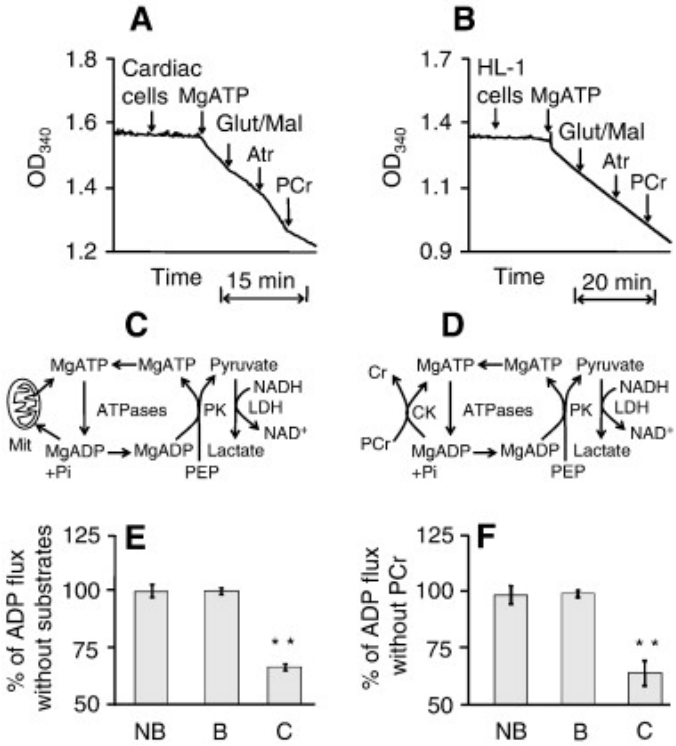

Fig. 9. A and B. Original recordings demonstrating direct transfer of ADP from ATPases to mitochondria and coupling of MM-CK to CaMgATPases in permeabilized cardiomyocytes isolated from rat ventricle and NB HL-1 cells, respectively. C and D. Reaction schemes in the spectrophotometric cuvette. Additions: Cells – 0.01–0.03 mg protein/ml (100,000–300,000 cells/ml), MgATP – 2 mM MgATP, Glut/Mal – 10 mM glutamate + 2 mM malate, Atr – 0.1 mM atractyloside, PCr – 20 mM PCr. **E. Absence of direct transfer of endogenous ADP from ATPases to mitochondria in HL-1 cells.** The columns demonstrate the averages of the measurements in different groups. NB – NB HL-1 cells, $n = 4$, B – B HL-1 cells, $n = 6$, C – rat cardiomyocytes as control, $n = 3$. * – $p < 0.005$ as compared to NB or B HL-1 groups. **F. Lack of coupling of CK to ATPases to in HL-1 cells.** The columns demonstrate the averages of the measurements in different groups. NB – NB HL-1 cells, $n = 3$, B – B HL-1 cells, $n = 4$, C – rat cardiomyocytes as control, $n = 3$.** – $p < 0.005$ as compared to NB or B HL-1 groups. Paper VI.

These findings suggest that diffusion of ADP is not significantly restricted and CK-mediated system of energy transfer may not function in HL-1 cells, hence, energy metabolism in HL-1 cells might be organized differently from that observed in adult cardiac cells. Indeed our further studied revealed several novel observations characterizing this situation. Firstly, no direct channeling of adenine nucleotides between mitochondria and ATPases could be observed in HL-1 cells. As shown in Fig. 9A, in accordance to earlier results (above, Fig. 2),

activation of mitochondrial OXPHOS by the addition of respiratory substrates significantly attenuated the ADP flux for the PK-PEP system, showing direct channeling of ADP from ATPases to mitochondria in normal cardiomyocytes. In contrast, mitochondria were unable to capture ADP in both types of HL-1 cells, which indicates the absence of those structural and functional interactions between mitochondria and ATPases that enable direct channeling of adenine nucleotides in these cells (Figs 9B and 9E).

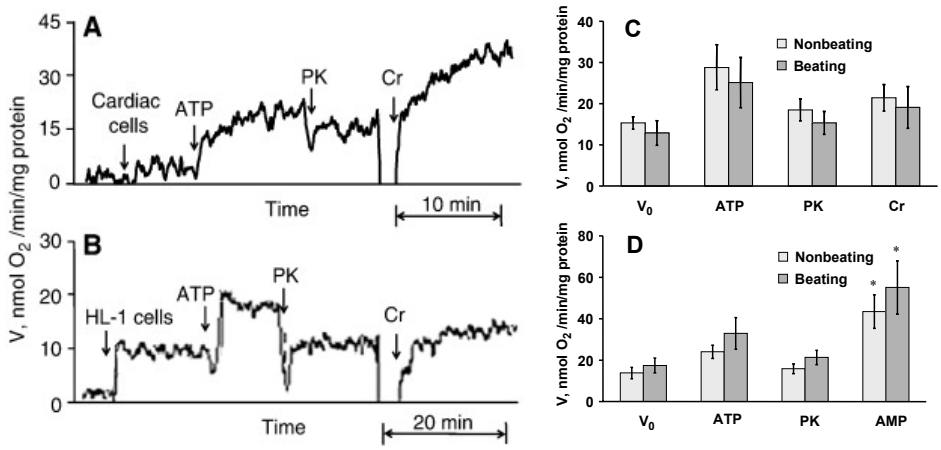

Fig. 10. Oxygraphic analysis of stimulation of OXPHOS by mitochondrial kinases via local ADP production. A. Original recording for assessing the effects of creatine on OXPHOS in saponin-permeabilized cardiomyocytes isolated from rat ventricle. The cardiomyocytes were permeabilized with 50 μg/ml saponin as shown in Materials and methods before transferring to oxygraph's chamber. After registration of the baseline respiration rate the following additions were made: ATP – 2 mM MgATP, PK – 20 IU/ml PK, Cr – 20 mM creatine. B. Original recording for assessing the effects of creatine on OXPHOS in permeabilized B HL-1 cells. The HL-1 cells were incubated with saponin as shown in the legend of Fig. 6, but in the presence of 5 mM PEP. C and D. The averages of measurements of respiratory rates of the experiments for testing the local ADP production by mitochondrial CK and AK2, respectively, in B (n = 5) and NB (n = 4) HL-1 cells. Additions in D are similar to that in B and C except that 2 mM AMP was added after stabilization of respiration with PK. V0 basal respiration of HL-1 cells incubated in Mitomed medium with 5 mM PEP. – $p < 0.05$ as compared to respiration rate before 2 mM AMP addition. Paper VI.

Secondly, the HL-1 cells differ from adult cardiomyocytes yet by the absence of coupling of mi-CK to ANT (Anmann et al., 2006). Indeed, as shown in Fig. 10) in these cells creatine was ineffective in activating respiration in the presence of the PK capable to trap all ADP produced by ATPases, in contrast to that in normal cells where it strongly activated respiration despite the presence of PK+PEP system. At the same time the HL-1 cells exhibited a strong activation of respiration by AMP indicating functional coupling between mi-AK2 and

ANT (Fig. 10D). Thus, it was not mi-CK but mi-AK2 isoform that was coupled to OXPHOS because only the latter enzyme could increase local MgADP in the intermembrane space not available to exogenous ADP-trapping system. For HL-1 cells, a very low level of mi-CK expression (Paper VI) associated with its negligible functional activity (Fig. 11) may be one reason for missed coupling, as not enough mi-CK is available for interacting with ANT at the outer aspect of mitochondrial inner membrane. At the same time the HL-1 cells express large amounts of mi-AK2 (Paper VI; Fig. 11A) that underlies its effective coupling to OXPHOS.

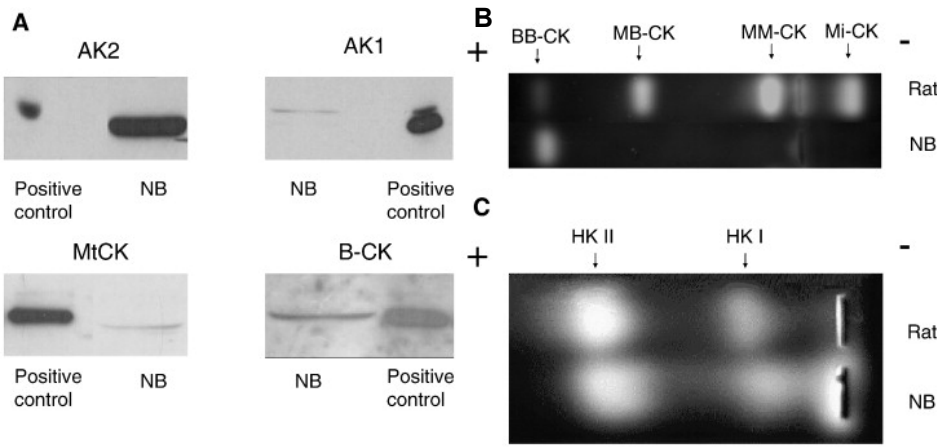

Fig. 11. A. Immunoblots tested with polyclonal rabbit antibodies against AK1, AK2, MtCK and affinity-purified chicken IgY antibodies against B-CK. Homogenized NB HL-1 cells containing 50 μg total protein were used as a sample (NB). To verify B-CK and MtCK, recombinant human proteins (approximately 1 μg) expressed in E. coli were applied as positive controls. For AKs, protein from the cytosolic or mitochondrial fractions of the rat heart was used as positive controls for AK1 or AK2, respectively. **B. Agarose gel electrophoresis of CK isoforms**. Homogenate of NB HL-1 cells was used as a sample (40 μg total protein) (NB). For control, a sample of the homogenate of rat ventricular myocardium (10 μg total protein) was run (Rat). **C. Agarose gel electrophoresis of HK isoforms.** Homogenate of NB HL-1 cells was used as a sample (20 μg total protein) (NB). For control, a sample of the homogenate of rat ventricular myocardium (95 μg total protein) was run (Rat). Paper VI

Thirdly, we found that the BB-CK, although abundantly expressed (Paper VI; Fig. 11A,B) was not coupled to ATPases. This was revealed in the same experiment and on the same sample used to evaluate the direct adenine nucleotide transfer. In HL-1 cells addition of PCr exerted no effect on ADP flux generated by ATPases and monitored by the PK+LDH system (Fig. 9 B, F). In contrast, similarly to earlier studies (Saks et al., 2006, Saks et al., 1984), Fig. 9 A, F shows that PCr markedly attenuated the ADP flux (by 37%) in cardiac

cells, which means that this portion of ADP generated by ATPases became unaccessible for exogenous PK+PEP system for it was rephosphorylated at the expense of PCr within the complex between MM-CK and CaMgATPases.

In normal cardiac muscle cells, PCr strongly attenuates the ADP flux because it triggers phosphorylation of ADP by MM-CK in a microcompartment between the CK and ATPases not accessible to exogenous PK (Saks et al., 1984). For such interaction, the structural closeness of MM-CK to CaMgATPases is required, like it occurs in sarcomeres where the CK is attached to the M-line near myosin heads (Hornemann et al., 2000). The reason for lacking functional coupling between the BB-CK and CaMgATPases in HL-1 cells is presently unclear. It is unlikely that coupling failed due to insufficient quantity of BB-CK because the cellular CK activity in direction of ATP formation equaled approximately to CaMgATPase (Paper VI). More plausible explanation would be that the BB-CK reminds unbound to specific sites near individual ATPases that hinders interaction between these two enzymes. Altogether, the current data allow to state that the CK mediated energy transfer is inoperative in HL-1 cells. In these conditions, strong expression of mi-AK2 isoform (Paper VI) associated with its effective coupling to OXPHOS (Fig. 10) may represent a compensation for inadequate mi-CK in HL-1 cells.

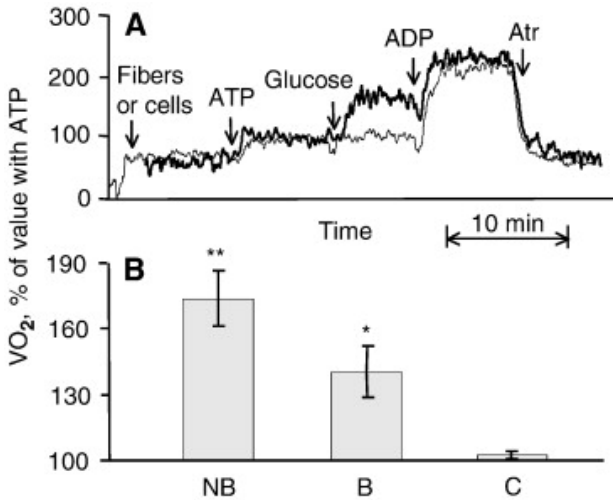

Fig. 12. Oxygraphic analysis of coupling of HK to OXPHOS in permeabilized HL-1 cells and rat ventricular cardiomyocytes. **A.** Original recording. Additions: ATP – 0.1 mM MgATP, Glucose – 10 mM glucose, ADP – 2 mM MgADP, Atr – 0.1 mM atractyloside. Thin line – permeabilized rat ventricular fibers; thick line – B HL-1 cells. **B.** The averages of stimulation of respiration in NB (n = 6) and B (n = 4) HL-1 cells (NB and B, respectively) and adult cardiomyocytes (C) relative to respiration rate with 0.1 mM MgATP. * – $p < 0.01$; ** – $p < 0.001$ compared to cardiac fibers. Paper VI.

Fourthly, we found strong activation of respiration by glucose in permeabilized HL-1 cells in the presence of limited amounts of ATP (Fig. 12). Several facts suggest that this phenomenon stems from specific interaction of HK isoforms with mitochondria. (i) Both HK1 and HK2 isoforms can bind to VDAC (porin) protein in the MOM in skeletal muscles and myocardium (Anflous-Pharayra et al., 2007, Parra et al., 1997). As a result, mitochondrially produced ATP is used to phosphorylate glucose, whereas ADP liberated by HK reaction is transferred back into the matrix to stimulate OXPHOS via porin connected to ANT (Anflous-Pharayra et al., 2007, Parra et al., 1997, Wilson, 2003). (ii) We found that after permeabilization of the cell membrane, the NB and B HL-1 cells still retained the HK in activities of 197.7±52.2 (n=4) and 112.8±4.3 (n=6) nmol/min/mg of protein ($p<0.05$), whereas Parra et al have shown that in permeabilized skeletal muscle cells all the detectable HK activity belongs to the enzyme bound to mitochondria (Parra et al., 1997). This evidence suggests that most of the cellular HK activity remained with mitochondria in permeabilized HL-1 cells. In support of that the higher HK activity in NB cells compared to B cells correlated with stronger stimulation of respiration in former cells (Fig. 12). (iii) Strong activation of respiration, registered in our conditions in the presence of 0.1 mM MgATP, which is far below the Km of HK for that nucleotide (0.5–1 mM (Wilson, 2003)) can be explained only by formation of complexes between mitochondria and HK that leads to endogenous ATP/ADP cycling thereby amplifying the respiratory response in spite of the presence of minute quantities of MgATP (Wilson, 2003).

Interaction of HK with mitochondria observed in this study may represent a key step of the HK-mediated energy transfer in HL-1 cells, because it promotes glycolysis that in turn provides ATP (e.g. from PK reaction) for ATPases and ATP-dependent ion channels (Dzeja and Terzic, 2003). This assumption is substantiated further by the observations that in muscle cells coupling of HK to OXPHOS increases with enhanced workload (Parra et al., 1997) and augments the capacity of glucose uptake (Anflous-Pharayra et al., 2007), and that the unidirectional flux of high-energy phosphoryls through glycolytic system can reach the rates equal to those of OXPHOS in muscle cells (reviewed by Dzeja and Terzic (Dzeja and Terzic, 2003).

Interestingly, like in HL-1 cells, the Km for ADP in the regulation of respiration is very low in one-day-old postnatal rat cardiomyocytes, when the mitochondria exhibit random localization in the cytoplasm and the contractile apparatus is not yet developed to the adult level. (Tiivel et al., 2000). In light of these findings and characteristics of the function of different kinases, the immortal HL-1 cells appear to represent such phenotype in which the intracellular energy metabolism has been regressed from the ICEU type of organization toward a less developed system characterized by a lack of control over mitochondrial function by cytoskeleton in a course of cellular remodelling under conditions of prolonged culturing.

1.1.3. Identification of localized diffusion restrictions for adenine nucleotides as a basis of intracellular compartmentation of energy metabolism (Paper I)

The phenomenon of high apparent Km for exogenous ADP in regulation of mitochondrial respiration compared to the isolated mitochondria has been observed in many laboratories (Joubert et al., 2000, Kuznetsov et al., 1996, Niggli, 1999, Saks et al., 2001, Seppet et al., 2001, Seppet et al., 1991, Veksler et al., 1995, Veksler et al., 1987). In cardiac cells, the value of that parameter can be decreased by limited proteolysis (Saks et al., 2001), hypercontraction of sarcomeres, and/or in conditions when OXPHOS is regulated by local ADP produced near ANT due to its coupling to mitochondrial kinases (Fig. 4). Furthermore, in contrast to adult cardiac cells, with "cristal-like" exactness of mitochondrial organization (Fig. 8), the HL-1 cells with entirely distinct intracellular arrangement show low Km for ADP, in association with other differences in the mechanisms of intracellular regulation of OXPHOS. From these data it is clear that high K_m for ADP in regulation of mitochondrial respiration *in situ* in skinned MGM fibers results not from specific morphological properties (e.g. diameter) of the muscle cell but rather from specific organization of the intracellular energy metabolism, which may be similar to other oxidative muscles (Kuznetsov et al., 1996, Seppet et al., 2001, Veksler et al., 1995). Therefore, we have hypothesized that in oxidative muscle cells mitochondria, ATPases and part of the cellular adenine nucleotides are compartmentalized into ICEUs (Saks et al., 2001, Seppet et al., 2001). By creating localized restrictions on diffusion of ADP, these proteins limit the access of exogenous ADP to mitochondria, which underlies low apparent affinity of mitochondria to this nucleotide (Saks et al., 2001, Seppet et al., 2001). As an important approach for supporting this hypothesis, we also have applied mathematical modeling. Thus, two different mechanisms of diffusion restriction for adenosine were assumed: one considering the uniform diffusion restriction for adenine nucleotides (DistICEU model, Fig. 13) and another assuming that diffusion of adenine nucleotides is restricted due to local diffusion barriers created by the borders of the ICEUs (localized or nonuniform diffusion restriction, SmallICEU model, Fig. 13). To reveal which of these assumptions is correct within the cellular context the experimental data were compared with the data obtained by mathematical modeling for the following processes (i) inhibition of endogenous ADP-stimulated respiration by exogenously added PK+PEP system, (ii) kinetics of oxygen consumption stabilization after the addition of 2 mM ATP or ADP, (iii) ADP concentration buildup in the medium after the addition of ATP, and (iv) ATPase activity with inhibited mitochondrial respiration. The results show that when the localized diffusion restriction in the space containing ATPases in the near proximity of the mitochondria, all the measurements were reproduced by the model. In contrast, the uniformly distributed restriction model could predict only few of the experiments, such as

the dependence on V_{O2} on exogenously added ADP or ATP and sensitivity of ADP dependent respiration to inihibition by PK+PEP system.

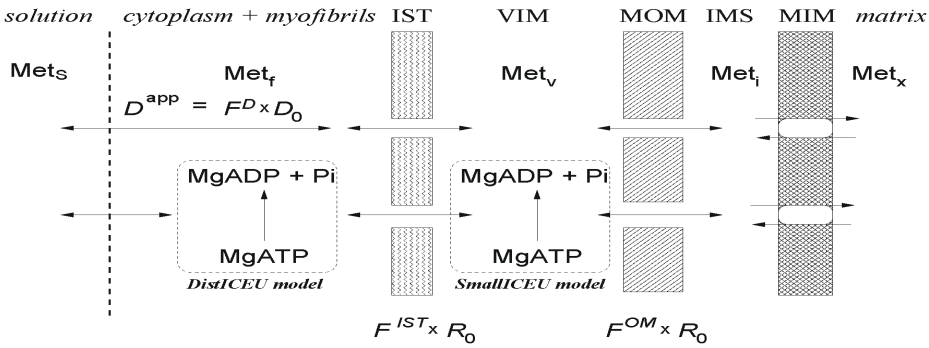

Fig. 13. Schematic presentation of ATP, ADP and Pi diffusion pathways from solution into mitochondria. Met_s, Met_f, Met_v, Met_i and Met_x – metabolite concentrations in the solution, in the myofibrillar and cytoplasmic compartment, in the vicinity of the mitochondria (VIM), in the mitochondrial intermembrane space (IMS), and in mitochondrial matrix, respectively. IST, MOM and MIM – intracellular structures between cytoplasm and VIM, mitochondrial outer and inner membrane, respectively. Depending on the model used, ATP is hydrolyzed either in VIM or myofibrils. Diffusion pathway is characterized by three model parameters: F^D, F^{IST}, and F^{OM}. Paper I

Thus, it is the muscle type-specific local diffusion restrictions, which being directly related to distinct structural organization of these cells underlie the differences in energy metabolism between the cardiomyocytes, glycolytic cells and HL-1 cells described above. In adult cardiomyocytes and possibly in other oxidative muscles, e.g. *m. soleus* and MGM, the mitochondria are tightly associated with adjacent sarcomeres into ICEUs arranged by cytoskeletal proteins, e.g. by desmin that cross-links the sarcomeres of neighboring myofilaments into register (Capetanaki, 2002). The cytoskeletal proteins also form barriers for adenine nucleotide diffusion at the level of MOM and within the ICEUS, which results in much higher apparent Km [200–400 μM) for exogenous ADP in regulation of OXPHOS in permeabilized cardiomyocytes than in isolated mitochondria (Anflous-Pharayra et al., 2007, Capetanaki, 2002, Saks et al., 2001, Seppet et al., 2001). To overcome diffusion limitations the ICEUs are equipped with a complex systems for energy transfer, mediated by different isoforms of CK and AK and direct channeling of adenine nucleotides, which activate ATPases and OXPHOS by increasing local ATP and ADP, respectively, (Anflous et al., 1997, Dzeja and Terzic, 2003, Saks et al., 2001, Saks et al., 2004, Saks et al., 1984, Seppet et al., 2001). Owing to this mechanism, the mitochondrial respiration becomes less dependent on the limited ADP flux from the cytoplasm,

this effect seen as a decrease in K_m for exogenous ADP after addition of creatine (Fig. 14). In contrast, in HL-1 cells with randomly arranged and dynamically changing mitochondrial networks (Fig. 8C,D) (Claycomb et al., 1998, Pelloux et al., 2006, White et al., 2004), the regulation of OXPHOS is entirely different: the Km for exogenous ADP is much lower (20–40 μM) than in cardiomyocytes (Anmann et al., 2006). The observation that exogenous PK+PEP system can easily reach the sites of ADP formation and to phosphorylate it there before it diffuses to mitochondria (Fig. 10) points to absence of significant diffusion barriers for adenine nucleotides in these cells as well. In glycolytic muscle cells, the diffusion restrictions are absent which is evident from very low apparent Km for ADP in regulation of respiration. In contrast to cardiac cells, these cells also exhibit large fluctuations in intracellular ADP (Kushmerick et al., 1992) in response to altered workload. These findings point to lack of the ICEU-like organization in glycolytic muscle cells.

2. Application of the ICEU concept in understanding the organization of energy metabolism in human muscle cell in conditions of norm and pathology (Papers II and IV)

Having proposed a hypothesis on the existence of the ICEUs in cardiac cells of different animal species, we were interested in characterizing the organization of energy metabolism in human muscle cells, with a special aim to reveal as to whether these cells are organized in the forms of ICEUs and whether the different pathological processes may affect the structure and function of the ICEUs.

2.1. Studies of energy metabolism in atrial muscle from patients with heart disease

In first part of studies the function of different systems of intracellular energy transfer was compared in atrial specimens isolated from patients with normal sinus rhythm (SR) and with AF.

To reveal the role of mi-CK in regulation of atrial mitochondria, the effect of 20 mM creatine on kinetics of stimulation of respiration by exogenously added ADP was assessed. Fig. 14 shows that creatine markedly increased the apparent affinity of mitochondria to ADP. This effect was similar to that in ventricular myocardium in rat and mice (Braun et al., 2001, Kuznetsov et al., 1996, Saks et al., 1998a). We also found that AMP stimulated respiration even without added ATP that means that the trace amounts of ATP bound to intracellular structures were sufficient for triggering the respiration-linked ADP-ATP cycling in the mitochondria. Since due to time-dependent increase in respiration at sub-maximal [AMP] the stationary [ADP] could not be adjusted and, therefore, estimation of coupling between mi-AK and ANT from the effects of AMP on Km^{ADP} was impossible, another protocol was used. After limited stimulation of

respiration with 50 μM ATP, 2 mM AMP markedly augmented the respiration due to ADP production by the mi-AK, and this process was abolished by AP_5A, a AK inhibitor. In general, there was a twice larger stimulation of respiration at 50 μM ATP with AMP than with creatine in all muscle groups. Earlier studies have shown that stimulation of the respiration by creatine or AMP in these conditions results from production of endogenous ADP produced near ANT owing to its functional coupling to mi-CK or mi-AK (Braun et al., 2001, Saks et al., 2001, Seppet et al., 2001). To reveal whether the similar mechanism play role in human atria, the effects of creatine and AMP on respiration were assessed in the presence of saturating concentrations of PK+PEP system effectively trapping the cytosolic ADP available (Kay et al., 2000, Seppet et al., 2001). The results show that both creatine and AMP strongly stimulated respiration even in the presence of PK+PEP system (Fig. 15), which means that it was not cytoplasmic ADP diffusing to ANT but endogenous ADP generated near the ANT that strongly stimulated the OXPHOS.

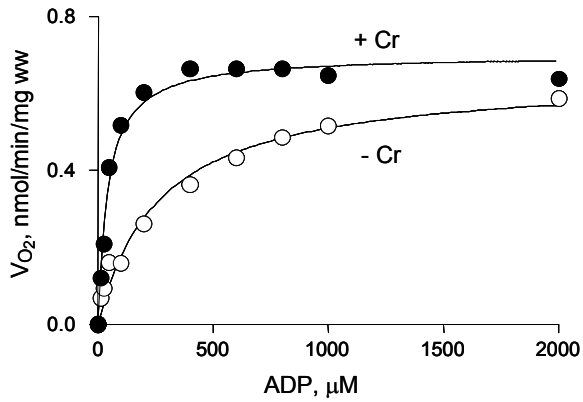

Fig. 14. Effect of creatine (20 mM) on V_{O2} vs [ADP] relationships in skinned atrial fibers of SR-patient. With and without creatine the hyperbolic relationship fitting with the Michaelis-Menten equation was obtained. Paper II

By developing a specific protocol we have earlier discovered that mitochondria and ATPases exchange ATP and ADP directly, independently of CK- or AK-phosphotransfer systems (Seppet et al., 2001). Therefore it was interesting to test whether a system of direct transfer exists in human atrial cells as well. To this end, all ATPases (CaMgATPases, for the medium contained both Ca^{2+}- and Mg^{2+}-ions) in SR and myofibrils were activated by addition of 2 mM of ATP and the ADP flux produced by ATPases was monitored by coupled PK+LDH reaction. In these conditions launching of OXPHOS by addition of respiratory substrates resulted in rapid decrease in the flux, through PK+LDH system whereas atractyloside exerted the opposite effect. This change indicates that

mitochondria effectively competed with powerful PK+PEP system for ADP released from ATPase reactions. Hence, there exists indeed a direct channeling of adenine nucleotides between mitochondria and ATPases in human atrial muscle cells. The same experimental protocol allowed us also to assess the functional coupling between MM-CK and ATPases, simply by adding 20 mM PCr into the medium after atractyloside. It can be seen that PCr the ADP flux available to PEP+PK system immediately decreased by 66% compared to that without PCr. This means that MM-CK and ATPase reactions are functionally coupled to each other due to which ADP produced by ATPases is rephosphorylated by PCr, and therefore unaccessible to cytoplasmic PK.

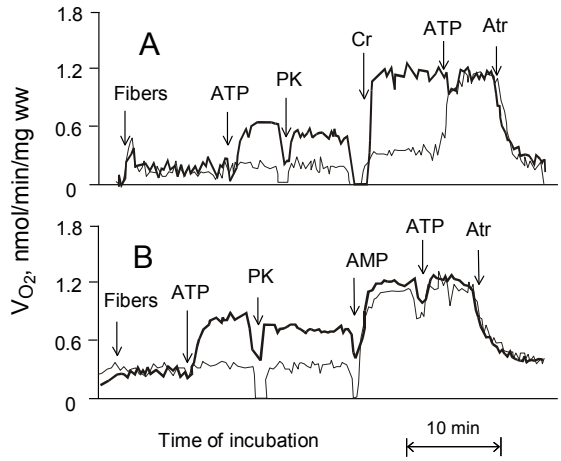

Fig. 15. Respirometric investigations of coupling of kinases to ANT in atrial fibers from the SR – patient. Additions: **A**, (thin line) – fibers, 50 μM ATP, 20 IU/ml PK, 20 mM Cr, 2 mM ATP, 0.1 mM atractyloside. Thick line – the same, but 2 mM ATP instead of 50 μM ATP. **B**, (thin line) – fibers, 50 μM ATP, 20 IU/ml PK, 2 mM AMP, 2 mM ATP, 0.1 mM atractyloside. Thick line – the same, but 2 mM ATP instead of 50 μM ATP. Note the effective control over the oxidative phosphorylation at the level of ANT (inner mitochondrial membrane) revealed by atractyloside. Paper II

Overall, these findings show that human atrial cardiomyocytes are similar to rat ventricles in that that they possess analogous networks of intracellular energy transfer, mediated by CK- and AK, and direct channeling of adenine nucleotides. At the same time the human atrial cells differ from those in rat atria, since the latter do not exhibit functional coupling between mi-CK and ANT, hence they lack the CK-mediated energy transfer pathway (Anflous et al., 1997, Vannier et al., 1996).

Considering the existence of different means of energy transfer it was interesting to assess their possible interaction in human atrial cells. Therefore, we studied the function of direct transfer system depending on the intracellular

ATP content and co-activation of CK- or AMP-mediated systems. The results show that at low [ATP] (50 μM), creatine produced much less local ADP than did AMP, because the effect of creatine on respiration was smaller compared to effect of AMP. Most interestingly, with either AMP or creatine, neither respiratory substrates nor atractyloside exerted effect on the ADP flux. Hence, contrary to that in the presence of 2 mM ATP alone, the direct transfer did not manifest at low [ATP]. When both the ATPases and CK were activated by 2 mM ATP and 20 mM creatine, the total ADP flux largely exceeded that in previous experiments, and the mitochondria became to control the ADP available for PK. Notably, now the direct flux was less (32 ± 2%) than in conditions when exclusively the ATPases provided ADP for mitochondria (55%, $p<0.001$). This finding suggests that in conditions of near physiological ATP levels the CK-mediated energy transfer interferes with direct channeling of adenine nucleotides between mitochondria and ATPases, e.g. as MM-CK consumes part of ADP produced by ATPases.

In our studies we addressed also the question as to whether the AF is associated with alterations in OXPHOS and in the intracellular energy transfer systems. Our studies did not reveal any change in energy transfer, however. In contrast, the muscle preparations from patients with AF showed increased succinate-dependent respiration (Table 1, Paper II) as compared to control. Since glutamate-dependent respiration did not change in parallel the limitation of electron flow at the level of complex I could take place. In parallel, increased proton leak was observed in AF atria. These changes might be related to impairment of the respiratory chain by ROS, as suggested by other studies (Arokoski et al., 2002, Claycomb et al., 1998, Kay et al., 1997a).

2.2. Studies on human *m. gluteus medius* (Paper IV)

Besides the myocardium, *m. soleus* and few glycolytic muscles, a large variety of muscles, particularly in human, have not been assessed as yet in terms of mitochondrial regulation. Therefore, we undertook studies on human MGM, a muscle controlling stability of pelvis.

Figs. 16A,B show that in MGM of control patients, the kinetics of activation of mitochondrial respiration by exogenous ADP followed the Michaelis-Menten equation, with the mean value of apparent K_m for ADP of 282 ± 56 μM in the absence of creatine. This K_m value well corresponds to that in oxidative muscles such as heart and *m. soleus*, but largely exceeds that in glycolytic muscles and isolated mitochondria (10–20 μM (Kuznetsov et al., 1996, Veksler et al., 1995)). MGM of control patients possesses a significant amount of mi-CK activity (4.3 ± 1.8% of total CK activity), which is close to that in *m. soleus* (6.8 ± 2.8, by (Anflous et al., 1997)). Likewise, creatine caused a four-fold decrease in apparent K_m for ADP (Fig. 16), this showing that mi-CK is functionally coupled to mitochondrial OXPHOS (Kuznetsov et al., 1996, Saks et al., 2004).

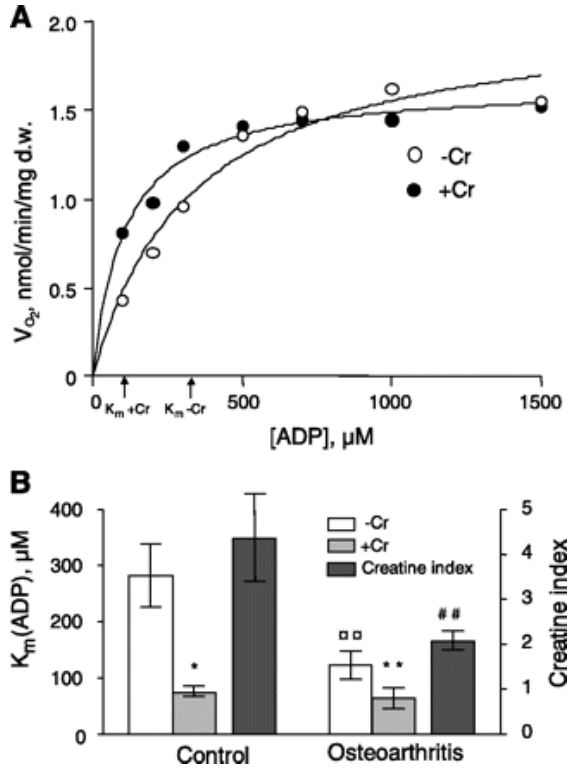

Fig. 16. The kinetics of regulation of mitochondrial respiration in skinned MGM by exogenous ADP and creatine. *A*: an example of the measurement in muscle fibers of control patient. Each data point indicates an increment in the rate of respiration ($\Delta\dot{V}O_2$) caused by ADP with given concentration over that prior to addition of ADP (v_0). *B*: data for control (n = 6) and OA (n = 16) muscle groups studied. *P < 0.05, **P < 0.01 compared with K_m for ADP (K_m; ADP) without creatine. ¤¤P < 0.01 compared with K_m for ADP in control group. ##P < 0.01 compared with creatine index in control group. Paper IV

High K_m for ADP in regulation of mitochondrial respiration and a decrease in that parameter by creatine *in situ* are the characteristic features of skinned oxidative muscle cells, probably rising from specific intracellular organization of the intracellular energy metabolism (Kuznetsov et al., 1996, Saks et al., 2001, Saks et al., 2004, Seppet et al., 2001, Veksler et al., 1995). Thus, based on similar kinetics of mitochondrial regulation in situ it seems conceivable to suggest that by its type of respiratory regulation and key enzyme activities the MGM represents a novel muscle belonging to the class of oxidative muscles. As discussed below (in relation to paper V), it is most likely the specific unitary organization of energy metabolism in the forms of ICEU like in other oxidative muscles that ensures precise regulation of mitochondrial ATP synthesis in

response to its use, thereby enabling chronically strong twitches of MGM for stabilization of the hip joint and pelvis during gait.

We found that OA is associated with significant changes in the cellular energy metabolism in MGM. First, increased sensitivity of mitochondrial respiration to ADP was observed, as K_m for ADP in regulation of respiration decreased in MGM more than twice and creatine exerted a diminished effect on that parameter (Fig. 16). Secondly, decreased total activities of AK and CK with major reduction in mi-CK fraction and a fast-to-slow MHC transition showing remodeling of the muscle tissue, were detected. These results can be interpreted as alterations in the structure and function of the ICEUs.

Importantly, application of the concept of ICEUs allows to outline the mechanisms underlying the pathology of the muscle cells. For instance, we found that K_m for ADP decreased in MGM more than twice and that creatine exerted a diminished effect on that parameter after development of OA (Fig. 16). Given the independence of K_m for ADP of muscle cell geometry (see above), these changes can not be attributed to the modest decrease in MGM fiber diameter (4–32%, depending on patients's age and fiber type) accompanying the muscle atrophy in patients with OA (Sirca and Susec-Michieli, 1980). It seems more likely that OA is associated with loosening of the cytoskeletal restrictions for ADP diffusion on the borders of or inside the ICEU, due to which this compound added exogenously reaches mitochondria more easily than in normal muscle, this resulting in decreased apparent K_m for ADP in the absence of creatine (Fig. 16B). An excess ADP flux from the medium/cytoplasm to the intermembrane space of mitochondria may inhibit PCr synthesis in mi-CK reaction, whereas diminished mi-CK activity may result in the same effect via impaired coupling to ANT. Clearly, both mechanisms should reduce the effectiveness of CK-energy transfer between mitochondria and ATPases in MGM. In these conditions the mitochondrial ATP synthesis becomes dependent on fluctuations of the cytoplasmic [ADP], which reduces both the mitochondrial PCr synthesis and the effectiveness of ATP usage at the ATPase sites (Saks et al., 2001) and therefore may contribute to decreased hip muscle strength (Arokoski et al., 2002) in OA patients. Since similar defects have been revealed under different diseases (e.g. ischemia/reperfusion impairment and heart failure (De Sousa et al., 1999, Kay et al., 1997b) and in oxidative muscles genetically devoid of dystrophin or desmin (Braun et al., 2001, Kay et al., 1997a) they probably represent a universal type of alterations resulting from disintegration of the ICEUs. It has been suggested that in case of failing CK system, activation of AK-phosphotransfer can play a compensatory role (Dzeja and Terzic, 2003). In diseased MGM, however, this mechanism may become also limited as suggested by decreased AK activity.

CONCLUSIONS

1. Mathematical modelling shows that in oxidative muscle cells diffusion restrictions of metabolites are not distributed uniformly within cardiac muscles, but are localized in certain areas, which supports the ICEU hypothesis.

2. Ca^{2+}-induced hypercontraction leads to decreased Km for ADP in regulation of respiration, this indicating increased availability of exogenous ADP for mitochondria during sarcomere shortening. At the same time, the direct transfer of ADP from ATPases to mitochondria decreased. These results offer a novel mechanism for controlling interaction of ATPases and mitochondria – via contraction-related alterations in localized restrictions for the intracellular diffusion of adenine nucleotides.

3. Differently from cardiomyocytes, in permeabilized HL-1 cells the OXPHOS is coupled to mitochondrial AK and HK but not to mitochondrial CK, and neither direct transfer of adenine nucleotides between CaMgATPases and mitochondria nor functional coupling between CK-MM and CaMgATPases is observed. Thus, whereas in cardiomyocytes mitochondria and CaMgATPases are organized into ICEUs which ensure effective energy transfer and feedback signaling between these structures via specialized pathways mediated by CK and AK isoforms and direct adenine nucleotide channeling, the ICEUs do not exist in HL-1 cells due to less organized energy metabolism.

4. Energy metabolism in human atria and MGM cells is organized similarly to that in oxidative muscles − in the form of the ICEUs. The ICEUs capture a part of the cytoplasmic pool of adenine nucleotides for being involved in energy transfer via CK- and AK-networks and direct exchange. The muscle preparations from patients with AF show increased succinate-dependent respiration and proton leak.

5. Pathogenesis of OA involves disintegration of the ICEUs in association with dysfunction of CK-phosphotransfer system and increased diffusion of ADP to mitochondria.

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SUMMARY IN ESTONIAN

Energia ülekande ja tagasiside regulatsioon oksüdatiivsetes lihasrakkudes

Hiljuti püstitati hüpotees, mille kohaselt oksüdatiivsetes lihasrakkudes moodus tavad mitokondrid ja ATPaasid komplekse, e. rakusiseseid energeetilisi üksusi (RSEÜd). RSEÜ-de raames on mitokondrid seostunud ATPaasidega spetsiaalsete energiatranspordi süsteemide vahendusel.

Praeguseks pole veel selge nende komplekside struktuuriline ja molekulaarne olemus. Hiljutistes eksperimentides tuvastatud adeniinukleotiidide otsene ülekanne mitokondrite ja ATPaaside vahel näitab, et adeniinukleotiidide difusioon südemelihasrakus on limiteeritud väga spetsiifilise rakusisese struktuurilise organiseerituse tõttu. Selgusetu on aga veel difusioonitakistuse ja seda põhjustavate rakusiseste struktuuride täpsem olemus, mis on seetõttu aktiivse uurimise objektiks.

Käesolevas uurimuses hinnati adenosiinfosfaatide difusioonitakistuse jaotumise erinevad võimalusi, võrreldes eksperimentaalseid kineetika mõõtmise andmeid erinevate matemaatiliste mudelite lahendustega. Vaatlused, et oksüdatiivsetes lihastes proteaaside põhjustatud rakustruktuuri desorganiseerumine või tsütoskeleti valkude defitsiit viib mitokondrite hingamise regulatsiooni suurenenud afiinsusele ADP suhtes, viitavad korrapärase rakustruktuuri olulisusele mitokondrite ja ATPaaside üheks kompleksiks seostumisel. Struktuurifunktsiooni vahelise sõltuvuse uurimine jätkub ka käesolevas töös. Käesoleval ajal ei ole veel selge, kas RSEÜ-d eksisteerivad ka inimese lihasrakkudes ja on muutunud mitmesuguste patoloogiliste seisundite korral, seetõttu uuritakse antud töös ka neid probleeme.

Konkreetsed töö eesmärgid olid:

1. Hinnata rakusiseste adeniinukleotiidide suhtes toimivate difusioonitakistuste osa lihasrakkude energeetilises metabolismis võrreldes matemaatilise modelleerimise tulemusi reaalsete eksperimentide andmetega.
2. Iseloomustada Ca^{2+} poolt põhjustatud hüperkontraktsiooni toimet mitokondriaalse hingamise regulatsioonile ja endogeense ADP otsesele ülekandele südamelihasrakkudes.
3. Iseloomustada energeetilist metabolismi normaalsetest südamerakkudest erinevate struktuuriomadustega HL-1 südamerakuliinis.
4. Iseloomustada kreatiin- ja adenülaatkinaaside vahendatud energiatranspordi süsteeme inimese südame kojas ja *musculus gluteus mediuses* ning näidata, et need süsteemid ja adeniinnukleotiidide otsene ülekanne on kompartmenteeritud nendes inimese kudedes RSEÜ-desse.
5. Iseloomustada energeetilise metabolismi muutusi inimese südame kojas kodade virvenduse tingimustes.

6. Hinnata energia ülekande süsteemide parameetreid *m. gluteus medius*-es sõltuvuses osteoartriidist.

Töö tulemused võimaldavad teha järgmisi järeldusi:

1. Reaalsete eksperimendiandmete matemaatiline modelleerimine näitab, et difusioonitakistused metaboliitidele pole jaotunud südamelihasrakus ühtlaselt vaid on lokaliseerunud, toetades seega RSEÜ hüpoteesi.
2. Ca^{2+}-põhjustatud hüperkontraktsioon vähendab hingamise regultsiooni Km-i ADP suhtes, väljendades eksogeense ADP muutumist mitokodritele kergemini kättesaadavaks sarkomeeri tugeva lühenemise kestel. Samas otsene ADP ülekanne ATPaasidelt mitokondritele väheneb. Need tulemused lubavad esitada uue mitokondrite ja ATPaaside funktsionaalse seose kontrollimehhanismi – lokaliseeritud difusioonitakistuste kontraktsioonist-sõltuvate muutuste kaudu.
3. Erinevalt kardiomüotsüütidest puudus permeabiliseeritud HL-1 rakkudes adeniinukleotiidide otsene ülekanne ATPaaside ja mitokondrite vahel ning oksüdatiivne fosforüülimise polnud seotud mitokondriaalse kreatiinkinaasi, vaid adenülaatkinaasi ja heksokinaasiga. Seega, erinevalt südamelihasrakkudest, kus mitokondrid ja ATPaasid on organiseeritud RSEÜ-desse, mis kindlustavad efektiivse energia ülekande ja tagasiside nende struktuuride vahel otsese adeniinnukleotiidide ülekande teel ning kreatiinkinaasi ja adenülaatkinaasi isoensüümide vahendusel, need üksüsed HL-1 rakkudes puuduvad, vähem organiseeritud energeetilise metabolismi tõttu.
4. Energeetiline metabolism inimese südame kojas ja *m. gluteus medius*-es on organiseeritud sarnaselt oksüdatiivsetele lihastele RSEÜ- vormis. See tähendab, mitokondrid ja ATPaasid moodustavad täpselt lokaliseeritud komplekse, kus toimub mitokondrite ja ATPaaside vaheline energia ülekanne ning tagasiside kreatiin- ja adenülaatkinaassete süsteemide vahendusel ning adeniinnukleotiidide otsese ülekande teel. Lihaspreparaate südamekodade virvendusega patsientidelt iseloomustab suurenenud suktsinaat-sõltuv hingamine ja prootonleke.
5. Osteoartroosi patogenees hõlmab RSEÜ-de osalist lagunemist, mis peegeldub kretiinkinaasse energiaülekande nõrgenemises ja eksogeense ADP suurenenud difusioonis mitokondrisse.

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PUBLICATIONS

CURRICULUM VITAE

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1979–1984	Faculty of Biology and Geography, University of Tartu
2002	Master's studies (external student), Faculty of Medicine, University of Tartu.
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Professional Employment

1999–2001	Senior laboratory assistant, Institute of General and Molecular Pathology, University of Tartu
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Special courses

February, 2005	NATO Advanced Research Workshop: Stress Induced Biochanges in the Heart: From Genes to Bedside. Antalya, Turkey
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Scientific work

Main research has addressed the mechanisms of coordinated interaction of consumption, transport and production of energy in oxidative muscles.

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Täiendus

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2007. juuni	Raku bioenergeetika suvekool: "Mitokondrid neurodegeneratiivsete- ja metabolismihaiguste korral", Tartu, Eesti

Teaduslik tegevus

Minu peamiseks uurimisvaldkonnaks on olnud energia tarbimise, transpordi ja produktsiooni koordineeritud toimimise mehhanismid oksüdatiivsetes lihastes.

Publikatsioonid

1. Eimre, M., Paju, K., Pelloux, S., Beraud, N., Roosimaa, M., Kadaja, L., Gruno, M., Peet, N., Orlova, E., Remmelkoor, R., Piirsoo, A., Saks, V., Seppet, E. (2008) Distinct organization of energy metabolism in HL-1 cardiac cell line and cardiomyocytes. Biochim Biophys Acta. 1777, 514–24, (Medline)
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