Structural and Functional Studies of Mitochondrial Respiration Regulation in Muscle Cells

MINNA VARIKMAA
Dissertation was accepted for the defence of the degree of Doctor of Philosophy in Chemistry on June 19, 2013.

Supervisors:  **Professor Valdur Saks, PhD** Laboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, Grenoble, France. Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia

**Professor Madis Metsis, PhD** Department of Natural Sciences, Tallinn University, Tallinn, Estonia

Opponents:  **Professor Erich Gnaiger, PhD** Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck

**Professor Maris Laan, PhD** Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu

Defence of the thesis: 29th of November 2013

Declaration:  
*Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.*

/Minna Varikmaa/

Copyright: Minna Varikmaa, 2013  
ISSN 1406-4723  
ISBN 978-9949-23-556-8 (publication)  
Lihasrakkude mitokondriaalse hingamise regulatsiooni struktuursed ja funktsionaalsed uuringud

MINNA VARIKMAA
## CONTENTS

INTRODUCTION .......................................................... 7
PUBLICATIONS ......................................................... 8
ABBREVIATIONS ...................................................... 9
REVIEW OF THE LITERATURE ..................................... 11
  1. Structure of muscle cells ........................................ 11
  2. Muscle contraction ............................................... 11
     2.1. Excitation-contraction coupling ......................... 14
  3. Muscle energy metabolism ..................................... 15
     3.1. Free energy conversion in mitochondrion ............... 16
  4. Frank-Starling law and paradox of metabolic homeostasis 16
     4.1. Regulation of mitochondrial respiration by Ca$^{2+}$ 18
  5. System level properties of energy metabolism ............. 19
     5.1. Structural and functional organization of energy metabolism 19
     5.2. Metabolic compartmentalization ......................... 20
     5.3. Phosphotransfer networks and creatine kinase system 22
     5.4. Regulation of respiration in vivo: theory of factor X 24
  6. Structure and function of tubulins ........................... 25
     6.1. Mitochondria interactions with micotubules and tubulin 28
AIMS OF THE THESIS ................................................. 30
MATERIAL AND METHODS ........................................ 31
  1. Preparation of biological materials ......................... 31
     1.1. Animals ......................................................... 31
     1.2. Preparation of permeabilized muscle fibers ............... 31
     1.3. Isolation of adult cardiomyocytes ........................ 31
     1.4. Isolation of mitochondria from cardiac muscle ........... 31
  2. Methods ............................................................ 32
     2.1. Oxygraphic measurements ................................... 32
     2.2. Quality tests of cardiomyocytes ............................ 32
     2.3. Measurements of concentration of cytochrome aa3 32
     2.4. Determination of protein content ......................... 32
     2.5. Determination of the rate of ATP and PCr production in CM by ion pair HPLC 32
     2.6. Immunofluorescence ......................................... 33
     2.7. Western blot analysis ........................................ 33
     2.8. Antibodies ...................................................... 34
     2.9. Confocal microscopy .......................................... 34
     2.10. Cell culture ................................................... 34
     2.11. Reconstitution experiments ............................... 34
     2.12. Determination of flux control coefficients (FCC) 35
RESULTS AND DISCUSSION ................................................................. 36
1. Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome (Article I) .................................................. 36
2. Mitochondria-cytoskeleton interaction: Distribution of β-tubulins in cardiomyocytes and HL-1 cells (Article II) ............................................. 40
3. Role of mitochondria-cytoskeleton interactions in respiration regulation and mitochondrial organization in striated muscles (Article III) ............................................................................................ 43
4. Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactosome (Article IV) ............................................................................................ 51
CONCLUSIONS ................................................................................. 54
REFERENCES .................................................................................. 55
ABSTRACT ....................................................................................... 72
AKNOWLEDGEMENTS .................................................................. 74
CURRICULUM VITAE ..................................................................... 76
ELULOOKIRJELDUS........................................................................... 79
LIST OF PUBLICATIONS ................................................................. 81
APPENDIX ....................................................................................... 83
PUBLICATION I ............................................................................... 83
PUBLICATION II ............................................................................. 103
PUBLICATION III ........................................................................... 117
PUBLICATION IV ........................................................................... 119
INTRODUCTION

Coordination of mitochondrial ATP production with utilization in cytosol is essential to certify muscle performance in variety of workloads. How highly oxidative muscles (e.g. heart, m. soleus) realize energetic communication between mitochondria and ATPases while maintaining constancy in their intracellular energy metabolites (e.g. ATP, PCr) has been a main query of muscle bioenergetics for decades. Yet, several important questions still remain unanswered. For example, it is still highly debated which metabolic signals mediate the energetic communication between mitochondria and ATPases in cardiac cells. Moreover, the regulator of mitochondrial outer membrane permeability for energy metabolites and the functional consequence of this regulation is not clear. Recent in vivo (Maldonado et al., 2010; Maldonado et al., 2013) and in vitro (Carre et al., 2002; Monge et al., 2008; Rostovtseva et al., 2008) studies suggest that tubulin might be involved in this regulation, however its role in muscle cells, particularly in mitochondrial respiration regulation, is largely uncovered.

Present study aims to tackle these problems by analyzing the mechanisms of mitochondrial respiration regulation and the functional and structural roles of tubulin in adult cardiac and skeletal muscle cells. In the literature overview muscle structure, energy metabolism, mitochondrial respiration regulation and tubulin system are reviewed. In the results and discussion part the kinetics of mitochondrial respiration regulation and analysis of tubulin distribution and function by respirometric, confocal microscopy and biochemical approaches are presented and discussed. The results of this study provide more in depth understanding of creatine dependent mitochondrial respiration regulation, reveal the importance of tubulin in this regulation and explain how metabolic homeostasis is achieved in highly oxidative muscles.
PUBLICATIONS


The author’s contribution to the publications:

I, II Author has carried out part of the experiments and participated in writing the manuscript.

III Author participated in planning and performing the experimental part. She carried out experiments with her coworkers and participated in writing the manuscript.

IV Author participated in writing the manuscript in collaboration with coworkers.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine 5´-diphosphate</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5´-triphosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>carboxyatractyloside</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyocytes</td>
</tr>
<tr>
<td>Cr</td>
<td>creatine</td>
</tr>
<tr>
<td>CTT</td>
<td>C-terminal tail</td>
</tr>
<tr>
<td>EDL</td>
<td>m. extensor digitorum longus</td>
</tr>
<tr>
<td>FCC</td>
<td>flux control coefficient</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>1,5-dihydro- flavin adenine dinucleotide</td>
</tr>
<tr>
<td>GR</td>
<td>m. gastrocnemius red</td>
</tr>
<tr>
<td>GW</td>
<td>m. gastrocnemius white</td>
</tr>
<tr>
<td>HKII</td>
<td>hexokinase II</td>
</tr>
<tr>
<td>ICEU</td>
<td>Intracellular Energetic Unit</td>
</tr>
<tr>
<td>IMS</td>
<td>mitochondrial intermembrane space</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MCA</td>
<td>Metabolic Control Analysis</td>
</tr>
<tr>
<td>MCC</td>
<td>metabolic control coefficient</td>
</tr>
<tr>
<td>MCU</td>
<td>mitochondrial Ca uniporter</td>
</tr>
<tr>
<td>MI</td>
<td>Mitochondrial Interactosome</td>
</tr>
<tr>
<td>MtCK</td>
<td>mitochondrial creatine kinase</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MMCK</td>
<td>muscle creatine kinase</td>
</tr>
<tr>
<td>MIM</td>
<td>mitochondrial inner membrane</td>
</tr>
<tr>
<td>MOM</td>
<td>mitochondrial outer membrane</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotineamine adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>dihydronicotineamine adenine dinucleotide</td>
</tr>
<tr>
<td>NB-HL-1</td>
<td>non-beating human atrial tumour cell lineage</td>
</tr>
<tr>
<td>PCr</td>
<td>phosphocreatine</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PIC</td>
<td>inorganic phosphate carrier</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca²⁺ release channel</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
</tbody>
</table>
TnC   troponin C
TnI   troponin I
TnT   troponin T
VDAC  voltage dependent anion channel
REVIEW OF THE LITERATURE

1. Structure of muscle cells

According to their structure and function muscle cells in vertebrates are classified into three following groups: cardiac, skeletal and smooth muscle cells. Skeletal muscles are attached to the bones and are responsible for all voluntary movements; cardiac cells are specialized for heart function and help to propel the blood throughout the body; smooth muscle cells line the blood vessel and various hollow organs apart from heart and are responsible for involuntary movements (Alberts et al., 2008). Due to the presence of interdigitating actin and myosin filaments, that give rise to characteristic cross-striated pattern, skeletal and cardiac muscles are jointly named as striated muscles.

Skeletal muscle cells are long multinucleated cells, which in adult human are typically 50 μm in diameter and several centimeters long. They are formed during the development by the fusion of myoblasts (in a range of tens of thousands!) into multinucleate syncytia (from Greek: σύν (syn) = "together" + κύτος (kytos) = "box, i.e. cell" (Squire et al., 2005; Abmayr and Pavlath, 2012). In contrast, heart muscle cells are significantly smaller about 50-100 μm in length and 10-25 μm in diameter in rat ventricle (Opie, 1984). They are branched and linked end to end by special structures known as intercalated disks, which provide mechanical and electrical continuity between cells (Squire, Al-Khayat et al., 2005). Cardiac muscle cells are alternatively called cardiomyocytes.

Muscle cells are invested by a cell membrane called sarcolemma which surrounds the crowded sarcoplasm. Almost two-third of bulk of the sarcoplasm is filled with contractile elements called myofibrils present in highly organized arrays. In between are sandwiched numerous mitochondria and calcium release units important for initiation of contraction and energy turnover. Nuclei are positioned right beneath the sarcolemma in skeletal muscles and at the center in cardiac cells. Sarcolemma penetrates into muscle cell interior as an extension of cell surface and forms series of tube-like invaginations called T-tubules. These are important for providing fast propagation of action potential, oxygen and metabolites throughout the cellular space (Soeller and Cannell, 1999).

2. Muscle contraction

The contractile apparatus of a muscle cell consists of bundles of partly overlapping thick and thin filaments, which form the myofibrils (Figure 1). In mammalian ventricle, myofibrils occupy 45-60% of the cell volume. This fraction is larger in skeletal muscle and smaller in atria (Bers, 2002). Myofibrils are subdivided by Z-lines into approximately 2 μm long compartments called sarcomeres, that represent one contracting unit of the myofibril. Thin filaments
are composed of actin protein, that extends from Z-line toward the center of sarcomere, while thick filaments are made of myosin protein, that resides at the center of sarcomere. In vertebrates, three main groups of actin isoforms, α, β, and γ have been identified. The prevalent form present in muscle cells is α-actin (Alberts, 2008). Under physiological conditions, monomeric form of actin, G-actin, polymerizes in presence of ATP into helical F-actin filament with a step of 13 actin monomers (Geeves and Holmes, 1999). According to X-ray diffraction analysis of crystals of actin-DNAsel (Kabsch et al., 1990), actin-gelsolin (McLaughlin et al., 1993), and actin profilin (Schutt et al., 1993), actin consists of four subdomains that surround the binding pocket for a divalent ion (Mg$^{2+}$ and Ca$^{2+}$) and nucleotide (ATP and ADP). Thick filaments are made up from bipolar polymers of motor protein, myosin II, that encompass two heavy chains with molecular masses of 200 kDa each and four light chains, two each of so called essential and regulatory light chains (ELC and RLC, respectively), with molecular mass 20 kDa (Gordon et al., 2000).

Under light microscope distinct areas of sarcomere are discernible. Area of light banding, that constitutes of actin filaments, is called I-band; intervening area of myosin and actin filaments is called A-band; area containing only myosin is called H-band, and region in the middle of sarcomere, where myosin is anchored, is called M-band (Figure 1)(Squire, Al-Khayat et al., 2005).

The precise architecture and functioning of myofibrils is supported by dozens of accessory proteins. The attachment of actin to Z-lines is mediated by protein α-actinin. Sensitivity of contraction apparatus to Ca$^{2+}$ is regulated by proteins tropomyosin and troponin C, I and T, which are bound to actin filaments and regulate the accessibility of myosin binding site on actin filaments. Two giant proteins titin and nebulin associate with actin and myosin respectively and support the myofilament structural arrangement (Alberts, 2008).

Muscle contraction is based on the cyclic interaction of actin and myosin filaments that result in their sliding past one another and shortening of the sarcomere. A first crucial step in understanding of this mechanism, was the discovery of interdigitating organization of actin and myosin filaments, made by Andrew Huxley and Ralph Niedergerke and by Hugh Huxley and Jean Hanson at 1950s (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). Following electron microscopy observations identified that active site of force transductions are represented by cross-bridges projecting from myosin filament to the actin filament. These projections correspond to heavy-meromyosin (HMM) subunit of the myosin molecule (Huxley, 1957). Since actin-combining activity and ATPase activity were already associated with this part of the molecule, it was clear that these cross-bridges represent the mechanical agents through which the force is transmitted (Szent-Gyorgyi, 1953; Huxley and Niedergerke, 1954; Huxley and Hanson, 1954).
Figure 1. A simplified model of muscle sarcomere and electronmicroscopic photograph of sarcomere ultrastructural organization. The sarcomere is comprised mainly of the thin and the thick filaments, and giant filamentous molecule titin. The thin filaments are anchored to the Z-line via α-actinin cross-link. The thick filament is centrally located in the sarcomere and constitute the sarcomeric A-band. The myosin heads, or cross-bridges, on the thick filament interact with actin during activation. Titin spans the half-sarcomeric distance from the Z-line to the M-line, thus forming a third sarcomeric filament. In the I-band region, titin is extensible and functions as a molecular spring that develops passive tension upon stretch (Ottenheijm et al., 2008).

The biochemical mechanism of ATP hydrolysis by myosin have been well described since 1970ies and is most commonly referred to as the sevenstep Bagshaw-Trentham scheme (Bagshaw and Trentham, 1974; Dantzig et al., 1992). The mechanism first involves rapid ATP binding to form actomyosin-ATP (AM-ATP, step 1) followed by the rapid dissociation to actin and myosin-ATP (M-ATP, step 2). Next there is a fast and reversible cleavage of ATP on the myosin head (forming M-ADP-Pi, step 3). Under physiological activating conditions, there is a rapid reassociation (step 4) of actin and M-ADP-Pi forming a weakly bound A-M-ADP-Pi state that then isomerizes (step 5) to a more strongly bound AM-ADP-Pi state (a transition probably regulated by Ca^{2+}). The strongly bound AM-ADP-Pi has been hypothesized to isomerize to produce force and AM-ADP-Pi (lever arm motion, step 6) and may be stabilized in the strong binding force-exerting form by the release of Pi in step 7 (Figure 2) (Dantzig, Goldman et al., 1992). The cycle is same for skeletal and cardiac muscles, but the rate constants controlling cross-bridge intermediate transition vary (Gordon, Homsher et al., 2000).
2.1. Excitation-contraction coupling

Muscle excitation–contraction coupling, a term coined in 1952 by Sandow, is a process from electrical excitation of the muscle cell to its contraction (Sandow, 1952). Although significant differences exist between skeletal and cardiac muscle, the general scheme of excitation-contraction coupling is similar. Contraction is initiated with the electrical excitation of the surface membrane, that in cardiac cells is mediated by pacemaker cells and in skeletal muscle by motoneurons. This leads to the action potential (AP) propagation along the surface and along the transverse tubules (T-tubules) as a wave of depolarization. Action potential activates the depolarization-activated Ca\(^{2+}\) channels in plasma membrane that triggers the inward current of Ca\(^{2+}\). Ca\(^{2+}\) entry triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). Increase in free intracellular levels of Ca\(^{2+}\) concentration, results in Ca\(^{2+}\) binding to the Ca-binding subunit of thin filament protein troponin C, exposure of myosin binding site on actin filament and activation of power stroke. For relaxation to occur intracellular Ca\(^{2+}\) concentration must decline enough to allow Ca\(^{2+}\) to dissociate
from troponin C (Bers, 2002). Four major classes of Ca$^{2+}$ transporters mediate the removal of Ca$^{2+}$ from the cytoplasm: 1) SR Ca-ATPase pump, 2) sarcolemmal Ca-ATPase pump, 3) sarcolemmal Na/Ca exchange and 4) mitochondrial Ca uniporter (Bers, 1991; Bassani et al., 1992; Baughman et al., 2011; De Stefani et al., 2011).

3. Muscle energy metabolism

The main part of energy transactions in muscle cells are related to the acto-myosin crossbridge cycling and removal of Ca$^{2+}$ from the sarcoplasm. The chemical driving force for these processes is ATP hydrolysis, carried out by specialized ATPases that transduce chemical energy into mechanical, osmotic and electrical work. Owing to the small reserves of ATP (e.g. 5 µmol/g wet wt in heart) in muscle cells and their high turnover rates (~0.5 µmol*g*wet wt$^{-1}$*s$^{-1}$ at rest in heart), sufficient only for few seconds of muscle work, continuous replenishment of ATP reserves is required (Kushmerick, 2005). Three main routes are exploited by cell to accomplish this task: short-term supply *via* phosphocreatine (PCr) hydrolysis, medium-term supply *via* anaerobic glycolysis, and long-term supply *via* oxidative phosphorylation of glucose and fatty acids to water and CO$_2$. The energy content of ATP is not fixed and depends on the extent of displacement of ATP hydrolysis from equilibrium as expressed by a following equation:

$$\Delta G_{ATP} = \Delta G^0 + R*T*\ln\{(ATP)*[(ADP)/(Pi)]-1\}$$

where $\Delta G_{ATP}$ is the effective free energy change of hydrolysis and $\Delta G^0$ is the free change of hydrolysis under standard conditions (Nicholls and Ferguson, 2002). Under normal physiological conditions mitochondrial oxidative phosphorylation maintains ATP hydrolysis reaction far displaced from equilibrium, providing $\Delta G_{ATP}$ values over -60 kJ/mol in certain cells (Kammermeier et al., 1982). In addition, large phosphocreatine pools present in muscle cells allow to minimize the changes in $\Delta G_{ATP}$ level during the periods in which there is disequilibrium of ATP hydrolysis and synthesis, through creatine kinase (CK) catalyzed transphosphorylation of ADP.

The main substrates for ATP production in muscles are free fatty acids (FFAs), glucose and glycogen. In heart 60–90% of the acetyl-CoA comes from oxidation of fatty acids, and 10–40% comes from the oxidation of pyruvate that is derived in approximately equal amounts from glycolysis and lactate oxidation (Gertz et al., 1988).
3.1. Free energy conversion in mitochondrion

In cardiac and oxidative skeletal muscle cells main part of free energy conversions and ATP synthesis are taking place in mitochondrion. Mitochondrion is double-membrane bounded powerhouse of eukaryotic cell that has arisen as intracellular symbiont of primitive cell (Muller et al., 2012). In typical mammalian cell mitochondrion carries out the complete oxidation of metabolites utilizing oxygen as terminal electron acceptor and harness the released energy for generation of ATP. This aerobic phase of catabolism is also called cellular respiration. It encompasses three main stages. In the first, organic molecules, already partly oxidized in cytosol, are transformed to yield two-carbon fragments in the form of acetyl group attached to coenzyme A, named acetyl-CoA. Next, acetyl-CoA are channeled into the citric acid cycle which carries out the stepwise removal of electrons and their transfer to NADH and FADH2 with the help of different dehydrogenases. In final step, these reducing equivalents fed electrons and protons to electron transport chain where they are transferred by electron carrying enzyme to terminal acceptor O2 with concomitant pumping of protons from mitochondrial matrix to intermembrane space. Altogether this process enables to generate protonmotive force between mitochondrial matrix and intermembrane space wherein flow of protons down to their electrochemical gradient drives the synthesis of ATP by ATP-synthetase (Alberts, Wilson et al., 2008).

Mitochondria occupy 30 to 40% of the intracellular volume of cardiac cell and synthesize 95% of ATP utilized by cell (Maack and O'Rourke, 2007). In skeletal muscles occupancy by mitochondria is significantly less, taking only 6-10% of cell volume in oxidative and 1% in glycolytic muscles (Barth et al., 1992; Ogata and Yamasaki, 1997; Picard et al., 2012). Under normal circumstances at rest, 60-90% of the acetyl-CoA which enters the tricarboxylic acid (TCA) cycle comes from the β-oxidation of FFAs, and 10-40% from the oxidation of pyruvate that is derived in almost equal amounts from glycolysis and lactate oxidation (Stanley et al., 2005). However, during conditions of increased metabolic demands, such as increased heart rate or blood pressure, a shift towards greater utilization of glucose is observed. Approximately 60-70% of ATP hydrolysis fuels contractile shortening, and the remaining 30-40% is primarily used for sarcoplasmic reticulum Ca2+-ATPase (SERCA2A) and other ion pumps (Stanley, Recchia et al., 2005).

4. Frank-Starling law and paradox of metabolic homeostasis

The Frank-Starling law describes the ability of heart to adjust its performance in response to the changes in end-diastolic volume (Starling, 1918; Katz, 2002). German physiologist Otto Frank described the relationship between diastolic volume and work in frog ventricle already at 1895 and Starling extended...
this theory further in his papers published between 1912 and 1918 (Chapman, 1960; Katz, 2002). Starling suggested that increased fiber stretch expose more active surfaces in the muscle such that “any increase in the extent of active surface increases energy of change” (Starling, 1918). This assumption received experimental proof in studies with frog skeletal muscles, which showed that maximal force that muscle can develop depends on the degree of the overlap between thick and thin filaments and is related to the cross-bridges that can form (Gordon et al., 1966). Consequent studies have shown that longer sarcomere length enhance myofilament Ca\(^{2+}\) sensitivity (Endo, 1972; Stephenson, 1982; McDonald et al., 1997).

![Figure 3](image-url)

**Figure 3.** Metabolic Aspect of Frank-Starling law and metabolic homeostasis.  
A Linear increase of oxygen consumption rates as a function of increased relative workload (fraction of maximal workload) (Williamson et al., 1976). B Constancy in cardiac intracellular PCr concentrations at different respiration rates. Experimental data is summarized from different studies (Neely et al., 1972; From et al., 1990; Wan et al., 1993). C Intracellular calcium homeostasis: stable calcium transients for different heart workloads (LVP stands for left ventricular pressure) of canine’s heart (Shimizu et al., 2002).

The metabolic basis of Frank-Starling law was discovered by Evan and Matsuoka who demonstrated that augmentation of cardiac work is accompanied by linear increase in oxygen consumption (Figure 3A) (Evans and Hill, 1914).
Thus, cardiac performance is directly related to the chemical transformations taking place in a cell.

4.1. Regulation of mitochondrial respiration by Ca$^{2+}$

Since the discovery of Ca$^{2+}$ accumulation into mitochondria by Hunter and Carafoli at 1960ies, parallel activation of contraction and respiration by Ca$^{2+}$ has become one of central viewpoints in explaining the metabolic aspect of Frank-Starling law (McCormack and Denton, 1990; Korzeniewski and Brown, 1998; Territo et al., 2000; Balaban, 2002; Carafoli, 2003; Maack and O'Rourke, 2007).

Ca$^{2+}$ cross mitochondria outer membrane (MOM) through VDAC and is transported to mitochondria matrix by mitochondrial calcium uniporter (MCU) (Baughman, Perocchi et al., 2011; De Stefani, Raffaello et al., 2011). Influx of calcium is facilitated by electrochemical gradient (-180 mV) that is set up by proton extrusion via respiratory chain (Bers, 2002). Calcium extrusion from mitochondria is mediated mainly via a Na/Ca antiporter (Crompton et al., 1976; Hayat and Crompton, 1985; Jung et al., 1995). Mitochondria in mammalian skeletal and heart muscle is shown in vivo to take up Ca$^{2+}$ during contraction and release it during relaxation, a process that appears to be important in some muscle types for regulation of excitation-contraction cycle (Robert et al., 2001; Rudolf et al., 2004; Shkryl and Shirokova, 2006). Due to mitochondria juxtapositioning with sarcoplasmic reticulum (SR) the synchrony between Ca$^{2+}$ release from SR and uptake by mitochondria is highly efficient (Sharma et al., 2000; Rudolf, Mongillo et al., 2004).

Apart from the role of mitochondria in cellular Ca$^{2+}$ buffering, energy conversion in mitochondria is also directly regulated by Ca$^{2+}$. Ca$^{2+}$ activates three Krebs cycle dehydrogenases (pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and the NAD-dependent isocitrate dehydrogenase) (Denton et al., 1978; McCormack and Denton, 1990; Hansford and Zorov, 1998), F$_{1}$F$_{0}$ATPase (Territo, Mootha et al., 2000) and Complex I (Brandes and Bers, 2002; Baniene et al., 2006).

Regulation of respiration only by Ca$^{2+}$ is however overruled by the estimates of the magnitude of changes in the respiration rate in vivo. Both experimental studies of calcium effects on mitochondria respiration in vitro (Territo, Mootha et al., 2000) and mathematical modeling of mitochondrial metabolism (Cortassa et al., 2003) show that changes in Ca$^{2+}$ concentration up to 600 nM can at maximum double respiration rate (Saks et al., 2006). Moreover studies with Ca$^{2+}$ probes have demonstrated that only minor changes in cytosolic [Ca$^{2+}$] accompany muscle twitch force several-fold increase (Figure 3) (Allen and Kurihara, 1982; Kentish and Wrzosek, 1998; Shimizu, Todaka et al., 2002; Cortassa, Aon et al., 2003). These findings contradict the assumption that more Ca$^{2+}$ is released from SR with increase in sacromere length and disagree with a principle formulated by O’Rourke, according to which variations of cytoplasmic [Ca$^{2+}$] have to
correspond to changes in workload, ATP consumption and respiration rate (Aon et al., 2003). Thus, theory of parallel activation of contraction and respiration by Ca\(^{2+}\) is in contradiction with Frank-Starling law of contraction. Finally Ca\(^{2+}\) stimulation of mitochondria function cannot compensate the need for the delivery of phosphate acceptors ADP and creatine from cytosol to mitochondria that are still needed for respiratory control (Chance and Williams, 1956).

5. System level properties of energy metabolism

5.1. Structural and functional organization of energy metabolism

Cardiac cells have intricate energetic infrastructure. Mitochondria are arranged in highly ordered fashion between the myofibrillar lattice and form structural and functional contacts with major ATP consumption sites at myofibrils, sarcoplasmic reticulum and sarcolemma. Rapid scanning confocal microscopy observations have revealed that adjacent mitochondria in cardiac cells are relatively immobile and fixed at the limits of sarcomere (Beraud et al., 2009). Tightly coupled complexes of mitochondria and ATPases represent the basic organization pattern of cardiac energy metabolism and are termed as intracellular energy units (ICEU) (Figure 4) (Saks et al., 2001). This concept was developed on the basis of information of cardiac cell structure and experimental data obtained in the permeabilized cardiac cells and fibers (Saks et al., 1994; Saks, 1998; Seppet et al., 2001; Seppet et al., 2006). Within ICEU, metabolic feedback is realized via compartmentalized phosphoryltransfer systems, e.g. creatine kinase (CK), adenylate kinase (CK) and direct ATP/ADP channeling (Kaasik et al., 2001; Seppet, Kaambre et al., 2001; Saks et al., 2010). Organization of ICEU is directed by distinct cytoskeletal proteins, e.g. tubulin, desmin, vimentin, plectin, which integrity has a direct impact on the fidelity of metabolic feed-back signalling (Kay et al., 1997; Seppet, Kaambre et al., 2001; Appaix et al., 2003; Anmann et al., 2006; Wilding et al., 2006).

Organization of energy metabolism into ICEU provides privileged communication between sites of ATP consumption and production in mitochondria. Comparison of various sources of ATP to support SR ATPases have shown that mitochondrially produced ATP is almost 30 times more effective than exogenous in supporting the calcium uptake by SR (Kaasik et al., 2001). Moreover, kinetic studies with cardiac and m. soleus fibers show that endogenously channeling ADP is only partly accessible to competitive ADP consuming system, pyruvate kinase/phophoenolpyruvate (PK/PEP), in case of MgATP activation (~20-40% decline in respiration rate) and totally inaccessible then respiration is activated by creatine (Seppet, Kaambre et al., 2001; Saks et al., 2003). Such privileged channeling is not seen then intracellular structure is disrupted by trypsin treatment or in isolated mitochondria, wherein 70-80%
A decline in respiration rate is seen upon PK/PEP addition (Saks et al., 2001; Seppet, Kaambre et al., 2001; Appaix, Kuznetsov et al., 2003; Guzun et al., 2009).

**Figure 4.** Functional scheme of the Intracellular Energetic Units of adult cardiac muscle cell. Each sarcomere is supplied by its own mitochondrion that forms structural and functional contacts with major ATP consumption sites at cytosol, e.g. SR (sarcoplamic reticulum), myofibrils, sarcolemma. The mitochondrial and cytosolic cycles of ATP/ADP are linked preliminary via creatine phosphotransfer network. The key system in energy transfer from mitochondria to cytoplasm is Mitochondrial Interactosome (MI), formed by ATP synthase, adenine nucleotides translocase (ANT), phosphate carriers (PIC), mitochondrial creatine kinase (MtCK), voltage-dependent anion channel (VDAC) with bound cytoskeleton proteins. The shaded area in the upper right corner shows the Calcium Release Unit (Saks et al., 2012). Reprinter with permission from Elsevier.

### 5.2. Metabolic compartmentalization

Dense cytoskeletal network, high concentration of macromolecules, presence of numerous organelles, and interactions between proteins and cellular structures significantly hamper the diffusion of metabolites between intracellular microdomains. This phenomenon is known as molecular crowding (Agutter et al., 1995). Physically, macromolecules occupy in between 5 to 40% of total cellular volume, with total concentration of macromolecules reaching up to 400 mg/ml, and even larger fraction of the total volume is unavailable to other
molecules with comparable size (Ellis and Minton, 2003). Molecular crowding is especially pronounce in muscle cells, which are tightly packed with myofibrils, numerous mitochondria and cellular organelles, and entangled by dense cytoskeletal network. Data obtained from $^{31}$P-NMR study on bullfrog biceps muscle have demonstrated that the diffusion coefficients of ADP and PCr are over 2-fold lower in muscle cytoplasm than in aqueous medium (Yoshizaki et al., 1990). This difference is even more pronounce in case of macromolecules (i.e. 22-540 kDa), reaching largely over 5-fold, as shown by fringe pattern photobleaching (MFPP) experiments (Arrio-Dupont et al., 2000).

Organization of energy metabolism into macro- and microcompartments interconnected via phosphoryltransfer routes represents one of the principal ways how muscle cells bypass the metabolic constraints imposed by molecular crowding. In cellular context, a term compartment is understood as „subcellular region of biochemical reaction kinetically isolated from the rest of the cellular processes” (Friedrich, 1974; Saks, Khuchua et al., 1994). Macrocompartments are related to the existence of cellular organelles, while compartments in the size of molecular dimension, are known as microcompartments (Friedrich, 1974; Saks, Khuchua et al., 1994; Saks et al., 2008).

Studies on ischaemic heart were among the first to hint, that metabolites are not homogenously distributed within the sarcoplasm, but are specifically compartmentalized at distinct cellular areas (Gudbjarnason et al., 1970; Neely et al., 1973). Specifically, it was found that under conditions of total ischaemia the concentration of PCr falls sharply (nearly 80% decline) in parallel with the cease of heart contraction, while ATP concentration remain almost stable decreasing only by 10% at the end of the first minute of ischaemia (Gudbjarnason, Mathes et al., 1970). Similarly, heart failure occurs then CK is inhibited with 2,4, dinitrofluorobenzene despite of the presence of 80-85% of cellular ATP (Gercken and Schlette, 1968). Other studies have shown that about 70% of cellular ATP can be removed by perfusion without any changes in contractile force if creatine and PCr are present at normal concentrations (Gudbjarnason, Mathes et al., 1970; Neely, Rovetto et al., 1973; Kupriyanov et al., 1984; Kupriyanov et al., 1991). Altogether, these results have led to a conclusion that energy metabolites are dynamically and spatially compartmentalized in cardiac cells (Saks, Beraud et al., 2008).

Microcompartments can arise from restricted diffusion of metabolites and/or from multienzyme complexes that determine either directly or indirectly the directionality of metabolite movement (i.e. vectorial metabolism) (Mitchell, 1979). These closely localized protein (e.g. enzymes, transporters, channels) clusters, that share similar ligands, are referred as functionally coupled (functional coupling=metabolite channeling+microcompartmentalization). For the direct metabolite transfer to occur, multienzyme systems (enzyme clusters) need to be physically associated, in which case intermediates are directly transferred from one catalytic site of an enzyme to the other without the intervening release to aqueous phase (Velot et al., 1997; Maughan et al., 2005).
Such, functionally coupled complexes have been identified for example among Krebs cycle enzymes and glycolytic enzymes (Malaisse et al., 1996; Malaisse et al., 2004). Cycling of ATP/ADP within the cardiac cell mitochondria is another well known example of metabolic compartmentalization. According to kinetic studies, this phenomenon is a result of two parallel events: functional coupling of MtCK with ANT and limited permeability of MOM for adenine nucleotides (Jacobus and Saks, 1982; Saks et al., 1995; Saks et al., 2008; Guzun, Timohhina et al., 2009). In isolated heart mitochondria, where MOM permeability for adenine nucleotides is high (apparent $K_m^{\text{ADP}}=13.9\pm2.6 \ \mu\text{M}$ of mitochondrial respiration), addition of competitive ADP consumption system (PK/PEP) results in partial inhibition of creatine activated respiration rate ($50\%$ decline in $V_{O_2}^{\text{max}}$), while remnant rate is maintained due to direct transfer of ADP from MtCK to ANT (Gellerich and Saks, 1982; Guzun, Timohhina et al., 2009). In contrast, the effect of PK/PEP on creatine stimulated respiration rate is totally absent in cardiac cells, where MOM permeability for adenine nucleotides is restricted (apparent $K_m^{\text{ADP}}=329\pm50 \ \mu\text{M}$) (Guzun et al., 2009). The functional significance of limited MOM permeability was revealed in comparative analysis of MtCK reaction kinetics between permeabilized cardiomyocytes in situ and isolated mitochondria in vitro (Jacobus and Saks, 1982; Saks, Belikova et al., 1995; Saks, Kaambre et al., 2008; Guzun, Timohhina et al., 2009). Accordingly, the apparent dissociation constant of MgATP from its binary and ternary complexes with MtCK, $K_{i_a}$ and $K_a$, is significantly higher in situ ($1.94\pm0.86 \ \text{mM}$ and $2.04\pm0.14 \ \text{mM}$) than in vitro ($0.44\pm0.08 \ \text{mM}$ and $0.016\pm0.01 \ \text{mM}$, respectively), while the apparent dissociation constant of Cr, $K_{ib}$ and $K_i$ ($2.12\pm0.21 \ \text{mM}$ and $2.17\pm0.4 \ \text{mM}$) is reduced 10-fold in situ in comparison with in vitro ($28\pm7 \ \text{mM}$ and $5\pm1.2 \ \text{mM}$) with no change in PCr affinity (Guzun, Timohhina et al., 2009). Thus, MOM limit selectively the diffusion of adenine nucleotides, while promote the affinity of MtCK for creatine.

5.3. Phosphotranfer networks and creatine kinase system

Fritz Lipman, a German-American biochemist, was among the first to notice the analogy between the energy-carrying adenine nucleotide system and electrical circuit. He introduced a concept of ‘adenylate wire’ to describe the mechanism how spatially separated energy transducing processes in cell are linked (Dzeja et al., 2002). In muscle cells, both direct and indirect pathways are exploited to signal of the changes in ATP handling in cytosol to the site of ATP regeneration in mitochondria. Indirect pathways encompass creatine kinase (CK), adenylate kinase (AK), AMP kinase (AMPK) and glycolytic isoenzymes, while direct pathway is represented by direct adenine nucleotide diffusion (DANC) (Seppet, Eimre et al., 2006). In healthy adult cardiac muscle cells nearly 80-88% of high-energy phosphoryl transfer is provided by CK reactions, as revealed in
$^{18}$O-assisted $^{31}$P NMR studies (Dzeja et al., 1996; Dzeja et al., 1999; Pucar et al., 2004).

CK/PCr system is an evolutionary ancient ATP buffering system, that has arisen already at the dawn of evolution of metazoa (Ellington, 2000). It belongs to a group of phosphagen systems that are found in cell types with high and variable rates of energy turnover and is widespread throughout the vertebrates and is present also in many lower chordates and invertebrates (Ellington, 2001). Although CK/PCr is the only phosphagen system present in vertebrates, additional 7 phosphagens with their corresponding kinases are known to exist in animal kingdom (Ellington, 1989). Cellular phosphagens function also to trap inorganic phosphate (Pi), which is one of the mechanisms to control glycogenolysis in muscles (Griffiths, 1982).

In vertebrate tissues four subunit isoform of CK are present and expressed in tissue-specific manner: two cytosolic forms, M-CK and B-CK (M standing for muscle, B standing for brain), and two mitochondrial MtCK isoforms (Mt standing for mitochondrial) (Wallimann et al., 1992). In vivo, M-CK and B-CK subunits combine to give the three typical dimeric cytosolic MM-, MB- and BB-CK isoenzymes from which MM-CK is rather specific to differentiated sarcomeric muscle, BB-CK is found in brain and in variety of other tissues (Eppenberger et al., 1967). In muscles, MM-CK activity is associated mainly with a M-band region at myofibrils (e.g. 2 IU/mg protein in rat heart fibers) (Ventura-Clapier et al., 1987; Stolz and Wallimann, 1998), where it binds through its N-terminal part, as shown in study of chimeric MM-CKs (Stolz and Wallimann, 1998). MtCK is accumulated specifically in mitochondria and is present in two isoforms, i.e. striated muscle specific, sarcomeric sMtCK, and ubiquitous MtCK (Jacobus and Lehninger, 1973; Scholte et al., 1973; Jacobus, 1975; Hall and Deluca, 1980).

One of the main functions attributed to CK system is temporal energy buffering (Wallimann, Wyss et al., 1992). In cardiac muscle PCr pool size is relatively large, i.e. 20-35 mM, and CK has high ATP regeneration capacity - maximal rate of ATP synthesis by CK exceeds the rate of ATP synthesis by oxidative phosphorylation in 12-fold (Fitch et al., 1974; Ronca-Testoni et al., 1985; Ingwall et al., 1990). Thus, CK reaction provides continuous and efficient replenishment of cytosolic ATP reserves. Other important functions attributed to CK include: „spatial energy buffer“ or „energy transport system“ (Bessman and Geiger, 1981), prevention of ADP accumulation in cytosol for the avoidance of ATPase inactivation (Iyengar et al., 1982; Iyengar, 1984), proton buffering to prevent local or global acidification of the cell due to ATP breakdown, and maintenance of appropriate ATP/ADP ratio at sites of ATP consumption and regeneration (Wallimann, Wyss et al., 1992).
In mitochondrion, MtCK is bound to the external surface of the inner membrane by cardiolipin in close proximity to the adenine nucleotide translocator (ANT) (Jacobus and Lehninger, 1973; Barbour et al., 1984; Jacobus, 1985; Muller et al., 1985). According to stoichiometric studies, MtCK and ANT are present in equimolar amount both in cardiac and skeletal muscles (Kuznetsov and Saks, 1986). The normal state of the MtCK in heart is an octameric (Marcillat et al., 1987; Schlegel et al., 1988; Furter et al., 1992) and its maximal activity of PCr production is equal to the rate of ATP production in oxidative phosphorylation (close to 1 μmol/mg/min at 30°C) (Saks et al., 1975; Jacobus and Saks, 1982). Due close juxtapositioning of MtCK to ANT, ATP transported from mitochondrial matrix by ANT is directly fed to the active site of MtCK, hydrolyzed wherein to ADP in conjunction with PCr synthesis, and channeled back to mitochondrial matrix (Gellerich et al., 2002). It has been suggested that MtCK reaction reduce the free energy of ATP synthesis due to continuous regeneration of ADP within mitochondria that maintains appropriate ADP/ATP ratio in vicinity to ATP-synthetase (Gellerich et al., 2002). Channeling of ATP and ADP within mitochondria through coupled MtCK and oxidative phosphorylation reaction result in amplification of metabolic signal and allows thereby mitochondria to respond to a small changes in cytosolic ADP levels relevant under physiological conditions (Wallimann, Wyss et al., 1992; Saks et al., 2012).

5.4. Regulation of respiration in vivo: theory of factor X

Introduction of cell permeabilization technique by Kummel et al. at 1988 to study the respiration kinetics in cardiac cells, led to a puzzling discovery that the affinity of mitochondria respiration for exogenous ADP differs over manifold between in vitro, in isolated cardiac mitochondria, and in vivo, in permeabilized cardiac cells (Kummel, 1988). This study, along with numerous following reports, showed that the value of apparent $K_m^{ADP}$ of mitochondrial respiration is over 20-30 timer lower in isolated mitochondria (10-20 μM) than in permeabilized cardiac cells and fibers (300-400 μM) (Saks et al., 1991; Saks et al., 1993; Kuznetsov et al., 2008). To elucidate the underlying cause of this phenomenon intracellular diffusion restrictions, activities of cellular ATPases and interaction of mitochondria with various cytosolic proteins have been considered.

One of the popular viewpoints have been that low affinity of mitochondrial respiration for ADP in cardiac cells is caused by a molecular crowding. However, many important discoveries disagree with this assumption and point that more specific cellular factors are involved instead. Firstly, the value of $K_m^{ADP}$ of mitochondria respiration is several-fold lower (10-20 μM) in glycolytic fast-twitch muscle fibers in contrast to oxidative ones, although the diffusion distance from periphery to fiber core and the extent of molecular crowding in similar in
both tissue types (Veksler et al., 1995; Kuznetsov et al., 1996). Secondly, the value of $K_m^{ADP}$ appears to have strong dependence on tissue metabolic phenotype. For instance, high $K_m^{ADP}$ value is a common feature of highly oxidative tissues such as neurons (100 μM) (Monge, Beraud et al., 2008), hepatocytes (250-300 μM) (Fontaine et al., 1995), m. soleus fibers (250-350 μM) (Veksler, Kuznetsov et al., 1995; Kuznetsov et al., 1996) and cardiac cells/fibers (200-350 μM) (Saks, Vasil'eva et al., 1993; Kuznetsov, Tiivel et al., 1996; Kay, Li et al., 1997). Thirdly, the extraction of one of the major constituents of myofibrils, myosin, by 800 mM KCl treatment, is shown to have no considerable impact on the value of apparent $K_m^{ADP}$ in cardiac cells (Kay, Li et al., 1997; Andrienko et al., 2003). Latter confronts also the involvement of ATPase activity in this matter. Finally, osmotic disruption of MOM (25-35 microM) in cardiac cells has been shown to decrease $K_m^{ADP}$ to the level observed in isolated cardiac mitochondria, pointing that diffusion restrictions origin specifically from MOM (Saks, Vasil'eva et al., 1993; Vasil'eva et al., 1993). In all, these results have led to a conclusion that low affinity of mitochondrial respiration for ADP is a result of limited permeability of MOM for ADP that is regulated by some unknown cytosolic protein, named “factor X” (Saks, Khuchua et al., 1994).

Accumulating data from literature suggests that limited permeability of MOM for ADP stem from mitochondria-cytoskeleton interactions. Convincing evidence in favor of this is that upon disruption of cytoskeleton by proteases (predominantly trypsin) in cardiac cells, over manifold decrease in apparent $K_m^{ADP}$ of mitochondrial respiration is seen, although mitochondrial respiration rate ($V_{O_2}^{max}$) and the intactness of MOM remains unaltered (Kay et al., 1997; Andrienko et al., 2003; Appaix et al., 2003). In addition, it has been shown that microtubule and plectin, but not desmin network, are most sensitive to trypsin treatment (Kay, Li et al., 1997; Appaix, Kuznetsov et al., 2003). While desmin knock-out mice display also mitochondria disarray and increased respiratory sensitivity to ADP (Milner et al., 2000), evidence for their direct interaction is missing. Recent in vitro and in vivo studies, as described in more detail below, favor the idea that microtubular (MT) system is specifically involved in this regulation.

6. Structure and function of tubulins

Tubulins are a diverse family of proteins present in all mammalian cells. They are major components of cellular cytoskeleton and participate in variety of essential cellular processes, such as mitosis, intracellular transport, and cell motility (Luduena, 1998). Tubulin is a heterodimer of two globular proteins, α- and β-tubulin that assemble in head-to-tail fashion to form a linear protofilament. Both α- and β-tubulin are acidic proteins with molecular weight 55 kDa (Wade et al., 2009).
Figure 5. Microtubule structure and dynamic instability. Microtubules are composed of stable α/β-tubulin heterodimers that are aligned in a polar head-to-tail fashion to form protofilaments (a). Microtubule wall constitutes of 13 parallel protofilaments (b). Assembly–polymerization and disassembly–depolymerization of microtubules (c) is driven by the binding, hydrolysis and exchange of a guanine nucleotide on the β-tubulin monomer. Polymerization is typically initiated from a pool of GTP-loaded tubulin subunits (c1), followed by GTP hydrolysis and generation of metastable, blunt-ended microtubule intermediate (c2), which might pause, undergo further growth or switch to the depolymerization phase. A shrinking microtubule is characterized by fountain-like arrays of ring and spiral protofilament structures (c3). The polymerization–depolymerization cycle is completed by exchanging GDP of the disassembly products with GTP (c4) (Akhmanova and Steinmetz, 2008). Reprinted with permission from Nature Publishing group.

Tubulin assembly into 13 (generally) protofilaments gives rise to a microtubule (MT), that is a hollow tube of about 25 nm in diameter. This process is accompanied by GTP hydrolysis associated with β-tubulin so that principal constituent of MT is ‘GDP-tubulin’ (Wade, Garcia-Saez et al., 2009). MT ends are structurally and functionally distinct: minus ends expose α-tubulin subunits and are often stably anchored, whereas plus ends, exposing β-tubulin subunits, are highly dynamic and switch between phases of growth and shrinkage (Figure 5) (Nogales and Wang, 2006).
In vertebrates, 8 isoforms of $\alpha$-tubulin and 9 isoforms of $\beta$-tubulin are currently known (Sullivan and Cleveland, 1986). Isoforms of $\alpha$-tubulin are designated as $\alpha_1$, $\alpha_2$, $\alpha_3/7$, $\alpha_4$, $\alpha_6$, $\alpha_8$ and $\alpha_T$, and of $\beta$-tubulins as $\beta_I$, $\beta_{II}$ (A, B), $\beta_{III}$, $\beta_{IVA}$, $\beta_{IVB}$ (also known as IIC), $\beta_V$, $\beta_{VI}$, and $\beta_{VII}$ (Sullivan, 1988; Luduena et al., 1992; Luduena, 1993; Luduena, 1998; Lajtha et al., 2007). Further five members of the tubulin superfamily, $\gamma$, $\delta$, $\epsilon$, $\zeta$, and $\eta$, have been identified to date, but are less abundant (Wade, Garcia-Saez et al., 2009). Isoforms of $\alpha\beta$-tubulin differ mainly in their C-terminal tail (CTT), that is on average 10-15 residues in length and strongly acidic (contains about 40% of the total protein charge) (Sackett, 2010). The structure of CTT can vary due to posttranslational modifications (PTM) as well as to sequence differences. This influence the binding of distinct proteins to the MT surface, including motor protein kinesin, that conduct mitochondria transport (Amos, 2004).

**Figure 6.** A tentative model of the restricted permeation block of VDAC by tubulin. Negatively charged C-terminal tail of tubulin penetrates into VDAC pore in its opens state through interaction with positively charged channel walls and induce partial blockage of the channel (Rostovtseva and Bezrukov, 2012). Reprinted with permission from Elsevier.

Tubulin isoforms appear to be functionally distinct and vary in their spatio-temporal expression pattern. For example, $\beta_{IV}$ tubulin is reported to associate constantly with axonemal microtubules (Renthal et al., 1993); $\beta_{III}$ tubulin plays a protective role against free radicals and ROS (Ferreira and Caceres, 1992; Gan et al., 2007; Guo et al., 2010) and tubulin $\beta_{V}$ is found to decrease microtubule assembly in cells where it is overexpressed (Bhattacharya et al., 2011). Spatial segregation of tubulin isoforms has been found also in cultured neuronal cells, where $\beta_{II}$- and $\beta_{III}$-tubulin preferentially incorporated into colchicine-stable and colchicine-labile microtubules, respectively (Falconer et al., 1992). $\beta_{II}$-tubulin displays relatively narrow tissue specific expression pattern, present mainly in heart, muscle, testis and brain (Narishige et al., 1999; Nakamura et al., 2003; Leandro-Garcia et al., 2010).
It is well established that MTs are exploited in cells to direct mitochondrial subcellular distribution and to regulate mitochondria motility, fusion and fission (Anesti and Scorrano, 2006). Recent experimental data point that apart from their role in controlling mitochondria subcellular organization, MTs participate also in regulation of oxidative phosphorylation (Monge et al., 2008; Rostovtseva and Bezrukov, 2008; Rostovtseva et al., 2008; Maldonado et al., 2010; Sheldon et al., 2010; Sheldon et al., 2011; Rostovtseva and Bezrukov, 2012; Maldonado et al., 2013). Interaction between tubulin and mitochondria can be either indirect, through adaptor proteins (e.g. kinesin, dynein), or direct, involving membrane lipids and variety of membrane proteins, mostly unknown (Sackett, 2010).

First evidence for the existence of direct mitochondria-tubulin interaction came from the study with purified liver mitochondrial membranes. By using radioligand binding assay, it was shown that tubulin binds to mitochondrial membranes in saturable, reversible and high affinity ($K_m=3-5 \mu M$) fashion (Bernier-Valentin and Rousset, 1982). Shortly thereafter, immunoelectron microscopy observations evidenced that tubulin positions in close vicinity to mitochondrion in cardiac cells (Saetersdal et al., 1990). Recent electrophysiological experiments with voltage dependent anion channel, VDAC (Rostovtseva, Sheldon et al., 2008), revealed the functional significance of this interaction. Of note, VDAC is the most abundant protein in MOM, that in mammalian exists in three distinct isoforms (Anflous et al., 1998), and governs the ATP/ADP flux across MOM (Colombini, 2004; Lemasters and Holmuhamedov, 2006). Reconstitution experiments with VDAC demonstrated that tubulin, at already nanomolar concentrations, induce partial closure of a channel and decrease thereby channel permeability to ADP. In addition, these experiments identified that permeability blockage is mediated by tubulin CTT, as its removal via subtilisin cleavage abolished the observed effect (Rostovtseva, Sheldon et al., 2008). The model, proposed on the basis of these results, involves penetration of CTT of tubulin into positively charged VDAC lumen, which shifts the channel ion selectivity toward more cationic (Figure 6) (Rostovtseva and Bezrukov, 2012). Further proof for direct tubulin-mitochondria interaction has been provided by co-immunoprecipitation experiments with normal and cancerous cell lines, evidencing interaction between VDAC and βIII-tubulin (Carre et al., 2002). Also incubation of 1 μM tubulin with isolated brain mitochondria is found to increase the apparent $K_m^{ADP}$ of mitochondrial respiration from 9 μM to 169 μM (Monge, Beraud et al., 2008). Finally elevation of free tubulin levels in variety of cancer cells has been shown to decrease mitochondrial membrane potential, suggesting that tubulin suppress metabolite exchange through MOM (Maldonado, Patnaik et al., 2010).

Interaction of VDAC with tubulin can be influenced by several distinct cellular factors. First, hexokinase-2 (HKII), highly expressed in many tumor
cells, is shown to bind to VDAC and may thus compete with a binding of tubulin (Robey and Hay, 2006; Mathupala et al., 2009). Secondly, in vitro phosphorylation of VDAC by GSK3β and cAMP-dependent protein kinase A (PKA) has been found to increase the on-rate of tubulin binding to VDAC (Sheldon et al., 2011). Co-immunoprecipitation and GST-based affinity pull-down evidenced that in mouse cardiac mitochondria VDAC interacts with pyruvate kinase C epsilon (PKCε) and cardiac specific expression of PKCε in mice was found to inhibit Ca^{2+} induced permeability transition pore (mPTP) opening (Baines et al., 2003). Other studies have reported that VDAC phosphorylation has cardioprotective effect (Baines, Song et al., 2003; Clarke et al., 2008; Javadov et al., 2009) and it reduce the entry of ATP into the mitochondria under de-energized conditions (Das et al., 2008). Finally electrophysiological experiments have shown that tubulin interacts only with a certain VDAC isoforms, such as VDAC-1 and VDAC-2, and not with VDAC-3 (Maldonado, Sheldon et al., 2013).
AIMS OF THE THESIS

The aim of this thesis was to gain more in depth understanding of the mechanisms of mitochondrial respiration regulation in muscle cells, and to elucidate the role of tubulin in mitochondria functional regulation.

In order to achieve this aim specific objectives of this study were:

1. Kinetic analysis of energy fluxes from mitochondria into cytosol and an assessment of tubulin involvement in regulation of energy flux exchange through MOM.
2. Determination of mitochondria specific tubulin isoform responsible for the regulation of MOM permeability for adenine nucleotides in cardiac cells.
3. Analysis of the mechanisms of mitochondrial respiration regulation and βII-tubulin distribution in phenotypically distinct skeletal muscles.
4. Metabolic Control Analysis of reactions in Mitochondrial Interactosome in skeletal muscles.
MATERIAL AND METHODS

Detailed description of materials and methods is provided in the publications of this thesis. Briefly, the following approaches were used in the present study:

1. Preparation of biological materials

1.1. Animals

Male Wistar rats weighing 150-200g were used in the experiments. The animals were housed at constant temperature (22°C) in environmental facilities with a 12:12h light-dark cycle and were given standard laboratory chow ad libitum. Animal procedures were approved by Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

1.2. Preparation of permeabilized muscle fibers

Permeabilized fibers were prepared according to the method described earlier (Saks et al., 1998; Kuznetsov et al., 2008). The animals were anaesthetized, decapitated, and muscles of interest were placed into a plastic Petri dish containing ice-cold isolation solution. The dissection of the muscle strips was carried out in 20 minutes under a microscope of a cold light source, through diverse smooth horizontal moves to obtain thin muscle-fibers bundles. Fibers were permeabilized with 50 mg/ml saponin treatment keeping the mitochondrial membranes intact.

1.3. Isolation of adult cardiomyocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase A (Roche) using the adaptation of the technique described previously (Saks, Belikova et al., 1991). Isolated cardiomyocytes contained 70–90 % of rod-like cells when observed under light microscope. In order to study the regulation of mitochondrial respiration in cardiomyocytes, the sarcolemma was permeabilized by saponin keeping the mitochondrial membranes intact (Saks, Veksler et al., 1998; Kuznetsov, Veksler et al., 2008). The permeabilization procedure was carried out at 25°C with 20 μg/mL saponin.

1.4. Isolation of mitochondria from cardiac muscle

Mitochondria were isolated from adult rat hearts as described (Saks et al., 1975).
2. Methods

2.1. Oxygraphic measurements

The rates of oxygen uptake were determined with high-resolution respirometer (Oxygraph-2K, from OROBOROS Instruments, Austria) in Mitomed solution (Kuznetsov, Veksler et al., 2008). Measurements were carried out at 25ºC; solubility of oxygen was taken as 240 nmol/ml (Gnaiger, 2001). The respiration rates were expressed in nmol of oxygen consumed per minute per nmol of cytochrome \( aa_3 \) or in nmol of oxygen consumed per second per mg of protein.

2.2. Quality tests of cardiomyocytes

In order to to ensure reliable results, regular quality tests of CM and isolated mitochondria preparation were performed as described (Saks, Veksler et al., 1998; Kuznetsov, Veksler et al., 2008). Only the mitochondria and cardiomyocytes meeting the requirements were used in kinetic experiments.

2.3. Measurements of concentration of cytochrome \( aa_3 \)

The contents of mitochondrial cytochrome \( aa_3 \) in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically as described (Monge et al., 2009). The differential spectrum (reduced versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 100 spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK).

2.4. Determination of protein content

Protein concentrations were determining using BCA protein assay kit (Pierce, USA).

2.5. Determination of the rate of ATP and PCr production in CM by ion pair HPLC

Determination of the rates of PCr synthesis in permeabilized cardiomyocytes \( in situ \) under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC, as described previously (Sikk et al., 2009). Separations of Cr, PCr and adenine nucleotides were performed by ultraperformance ion-pair chromatography (UPLC) on a 2.1×100 mm ACQUITY
UPLC HSS T3 C18 column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCr, and adenine nucleotides, respectively. The retention time for the reagents were, in minutes, 0.63 (Cr), 1.70 (PCr), 6.33 (AMP, traces), 6.95 (ADP) and 7.29 (ATP), all within ±0.01 min.

2.6. Immunofluorescence

For mitochondria labelling, cardiomyocytes and HL-1 cells were preloaded with mitochondria-specific fluorescent probe 0.2 μM MitoTracker Red™ and Green™ for 15 min at 37°C (Molecular Probes, Eugene, OR). Images were analyzed using Volocity software (Improvision, France).

Immunolabelling of cytoskeletal and mitochondrial proteins was performed on intact rat skeletal or heart left ventricular muscle fibers in suspension. Fibers were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 and blocked with 2% BSA PBS solution. For immunolabelling of VDAC heat-mediated antigen retrieval was performed before permeabilization. Fibers, treated with primary and secondary antibodies, were mounted in ProLong® Gold Antifade Reagent with DAPI (LifeTechnologies), deposited on glass coverslips and observed by confocal microscope.

2.7. Western blot analysis

Muscles samples were quickly removed, flash-frozen in liquid nitrogen and ground into fine powder with mortar and pestle. Samples were homogenized at room temperature in Tris/Triton X-100 lysis buffer, centrifuged at 15 000 g for 4 min and insoluble material was discarded. Free and polymerized tubulin were assessed with a Microtubule/Tubulin In Vivo Assay Kit (Cytoskeleton) according to the manufacturer’s instructions. Polymerized microtubule fraction was solubilized in Brinkley buffer containing 4M urea.

Electrophoresis was performed on the Mini Protean II from BioRad on in the Tris-tricine buffer solution and blotting of the unstained gels was performed on the Trans-Blot SD Semi-Dry Transfer Cell (BioRad) using PVDFmembranes (Millipore). Blots were probed with primary antibodies for β-tubulin isoforms, VDAC and MtCK. Immunoblots were developed using anti-mouse (1:45000, Abcam) and anti-rabbit (1:1000, Abcam) secondary antibodies conjugated to peroxidase (Abcam). Detection was conducted using chemiluminescence kit (SuperSignal West Dura Extended Duration substrate, Pierce).
2.8. Antibodies

Mouse monoclonal anti-βI-tubulin (WB 1/20 000, IF 1/1000; Abcam), mouse monoclonal anti-βII tubulin (WB 1/250, IF 1/250; Abcam), rabbit monoclonal anti-βIII-tubulin (wb 1/1000, IF 1/50; Abcam), mouse monoclonal anti-βIV-tubulin (WB 1/400, IF 1/1000; Abcam), rabbit polyclonal anti-β-tubulin (WB 1/500, IF 1/200, Abcam), rabbit polyclonal anti-MtCK (1:250; Abcam), rabbit polyclonal anti-β-tubulin (1:500; Abcam), polyclonal rabbit VDAC antibody serum (1:1000; kindly provided Dr. Catherine Brenner, Universite Paris-Sud, Paris, France), DyLight 488 goat anti-rabbit IgG (1:250, Abcam), Dylight 549 goat anti-mouse IgG (1:250, Abcam).

2.9. Confocal microscopy

The fluorescence images were acquired by Zeiss LSM 510 confocal microscope (Carl Zeiss) equipped with a Plan-Apofluar 63x/1.30 glycerol objective. Laser excitation 488 nm was used for DyLight 488 with emission detected through a 505- to 530 band-pass filter, DyLight549 was excited at 561 nm and detected through 575- to 615 nm band-pass filter. Processing of all confocal data sets were done with LSM Image Browser software, performing rotation, cropping, linear contrast adjustment, channel balancing and addition of scale bar. Images presented were copy–pasted from LSM Image Browser to Photoshop CS4 without further modifications.

2.10. Cell culture

HL-1 mouse atrial cardiomyocyte tumor cells were cultured on fibronectin (12.5 mg/ml)-gelatine (0.02%) coated flasks in Claycomb medium (Signa) supplemented with 10% foetal bovine serum, 2 mM L-glutamine (PAN Biotech GmbH), 0.1 mM norepinephrine (Sigma), 0.3 mM ascorbic acid (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) in a humid atmosphere of 5% CO2/95% air at 37°C.

2.11. Reconstitution experiments

Isolated and purified rat heart mitochondria (8 mg/ml) were incubated in Mitomed solution with 1 µM tubulin for 30 min at room temperature (22 °C). Thereafter, oxygraphic measurements were carried out in presence or absence of 20 mM creatine. Kinetics of respiration activation was analyzed by successive addition of ADP (0.005-0.01-0.02-0.05-0.1-0.2-0.5-1-2-3 mM). Assay medium
contained 0.2% of serum bovine albumin and 1 IU/ml apyrase from potato (Sigma-Aldrich) as an ADP regenerating system.

2.12. Determination of flux control coefficients (FCC)

FCC allows to estimate the control that a certain reaction exerts on the steady-state flux of a overall metabolic pathway. To estimate the flux control coefficients of oxidative phosphorylation in mitochondria the method developed by Groen et al for irreversible inhibitors was used (Groen et al., 1982). The enzyme is stepwise titrated with a specific inhibitor, as the amount of inhibitor tends to zero, the response of the flux to the inhibitor can be expressed in metabolic control analysis terms. The FCCs were defined as the variations in flux ($J$) when an infinitesimal change in the enzyme $i$ concentration or activity takes place (Fell, 1997). In practice, the infinitesimal changes in $v_i$ are undetectable, and hence measurable noninfinitesimal changes are analyzed. Groen and his coworkers have shown that for the case of irreversible specific inhibitor an estimate of the value of the flux control coefficient is defined by following equation:

$$C^i_{V_i} = \frac{(\Delta J/\Delta I)}{I_{\text{max}}/J_0}$$

where $(\Delta J/\Delta I)$ is initial slope of the stepwise inhibition of oxygen respiration graph, $I_{\text{max}}$ is the inhibitor concentration giving complete inhibition, and $J_0$ is the initial steady-state flux value (Moreno-Sanchez et al., 2008). The flux control coefficients in permeabilized skeletal fibers were determined by using graphical method described by Fell (Fell, 1992). Following inhibitors were used: carboxyatractyloside (CAT) for ATP/ADP transporter, oligomycin for ATP Synthase, rotenone for complex I, antimycin-A (ANM) for complex III, sodium cyanide (NaCN) for complex IV and 1-Fluoro-2,4-dinitrobenzene (DNFB) for MtCK.
RESULTS AND DISCUSSION

1. Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome (Article I)

Many controversies exist in the literature regarding the mechanisms of metabolic feedback regulation of mitochondrial respiration in cardiac cells during workload changes. For example, creatine kinase (CK) reaction and its non-equilibrium state is ignored in some modelling studies and coordination of ATP production in mitochondria with its consumption in cytosol is considered to occur predominantly via direct ATP/ADP channeling (Beard, 2005; Wu et al., 2008). Other studies propose that calcium is a main feedback signal, that matches ATP supply by mitochondria with its demands in cytosol (Wu, Zhang et al., 2008; Balaban, 2009). Finally, evidence has been received that both pathways – PCr shuttle and direct ATP diffusion – mediate in nearly similar efficiency the energetic crosstalk between mitochondria and cytosolic ATPases (Kaasik et al., 2001). To gain more in depth understanding of this matter, we estimated in this study quantitatively the effectiveness of MtCK reaction in the control of mitochondrial respiration in cardiac cells and assessed also the role of tubulin in mitochondrial respiration regulation.

To estimate the contribution of phosphocreatine (PCr) flux in energy transfer from mitochondria into cytoplasm, the ratio of PCr/O2 was determined with the use of respirometry and HPLC/UPLC techniques. The closer this value is to 6, a theoretical maximum of P/O2, the higher is the extent of PCr that is produced in coupled MtCK and oxidative phosphorylation reaction (Nicholls and Ferguson, 2002). Mitochondrial respiration was activated by addition of fixed amount of MgATP (1, 2, 5 mM) and 10 mM creatine, in presence of ADP trapping system, pyruvate kinase/phosphoenolpyruvate PK (20 IU/ml)-PEP (5 mM), to prevent the exhaustion of cytosolic PCr reserves (Gellerich, 1982). The samples of reaction mixtures were collected at 3, 6 and 10 min after activation of respiration by creatine and the accumulation of PCr and ATP was determined by HPLC/UPLC technique. PCr and ATP concentrations were calculated from corresponding peak areas on chromatograms.

As demonstrated in Figure 7, the rate of ATP production exhibits no significant change within the surrounding medium, while concentration of PCr increase in time in dependence of MgATP concentration. To ascertain the contribution of MtCK reaction to PCr synthesis, the rate of overall PCr production was subtracted from the rate recorded in conditions of inhibited (10 μM rotenone) oxidative phosphorylation (Figure 7B and C). At any MgATP concentrations, the value of PCr/O2 was close to 6, with average value 5.68±0.14 (Table 1). This is close to previously reported theoretical maximum of P/O2 ratio...
measured under conditions similar to those *in vivo* (Nicholls and Ferguson, 2002).

**Figure 7.** A The rate of ATP production B The rate of PCr production by mitochondrial and cytoplasmic creatine kinases in presence (solid lines) and absence (dashed lines) of active oxidative phosphorylation C The net rate of oxidative PCr production.

<table>
<thead>
<tr>
<th>ATP Concentration</th>
<th>$aV_{PCr}$ μmol mg⁻¹ protein min⁻¹</th>
<th>$bV_{O2}$ μmol mg⁻¹ protein min⁻¹</th>
<th>$V_{PCr}/V_{O2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM ATP</td>
<td>0.23±0.02</td>
<td>0.041±0.001</td>
<td>5.80±0.45</td>
</tr>
<tr>
<td>2 mM ATP</td>
<td>0.31±0.02</td>
<td>0.056±0.02</td>
<td>5.44±0.44</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>0.43±0.04</td>
<td>0.074±0.003</td>
<td>5.68±0.48</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>5.68±0.14</strong></td>
</tr>
</tbody>
</table>

$^aV_{PCr}$ rate of PCr production measured with the use of HPLC/UPLC

$^bV_{O2}$ rate of oxygen consumption

**Table 1.** Measured rates of PCr production, corresponding oxygen consumption and their calculated ratios for fixed ATP concentration in permeabilized cardiac cells.

The efflux of PCr and adenine nucleotides from mitochondria occurs through MOM protein VDAC, which permeability for anions is regulated
presumably by tubulin (Monge, Beraud et al., 2008; Rostovtseva and Bezrukov, 2008; Rostovtseva, Sheldon et al., 2008). To test, if tubulin is able to restore low MOM permeability for ADP in isolated heart mitochondria, the kinetics of respiration regulation was assayed in the absence (control) or presence of 1 μM tubulin. As shown in Figure 8, in control, the apparent $K_m^{ADP}$ of mitochondrial respiration is very low, equal to $11 \pm 2$ μM, while upon addition of tubulin two populations of mitochondria with different $K_m^{ADP}$ emerge, one with very high value, equal to $330 \pm 47$ μM, and other with the value close to control, $9 \pm 5$ μM. Upon addition of 20 mM creatine, the value of apparent $K_m^{ADP}$ decreased to $23 \pm 6$ μM, similarly to that seen in intact permeabilized cardiac cells (Figure 8). According to previous respirometric studies, creatine increase the affinity of mitochondrial respiration for ADP due to activation of coupled MtCK reaction, that facilitate intramitochondrial ATP/ADP cycling (Saks et al., 1991; Saks et al., 1998; Meyer et al., 2006; Guzun, Timohhina et al., 2009).

Figure 8. Comparison of the apparent $K_m$ values of exogenous ADP in presence of tubulin and/or creatine.

High PCr/O₂ ratios observed for all tested MgATP concentration demonstrates that coupling of MtCK reaction with oxidative phosphorylation is highly efficient – almost all ATP synthesized within mitochondrial matrix is utilized in MtCK reaction for PCr generation. Thus, in contrast to previously proposed idea (Kaasik, Veksler et al., 2001; Joubert et al., 2008; Kuum et al., 2009), these results clearly show that under physiological conditions, when creatine activation is present, direct adenine nucleotide channeling between mitochondria and ATPases is only marginal. Earlier kinetics measurements have shown that affinity of MtCK for creatine in permeabilized cardiac cells is increased in comparison with isolated mitochondria (over tenfold decrease in the apparent $K_m^{ADP}$) and disruption of intracellular structure in permeabilized cardiac cells or isolation of mitochondria reduce creatine stimulated respiration rate in presence of PK/PEP system nearly 50% (Guzun, Timohhina et al., 2009). Thus, permeability restrictions for ADP are essential for facilitating coupling of MtCK reaction with oxidative phosphorylation and for enhancing intramitochondrial ATP/ADP cycling. Interestingly, the affinity for PCr is not changed between in situ and in vitro while differ substantially for MgATP, implying that restrictions
at MOM are selective for adenine nucleotides and favor thereby the control of respiration over creatine kinase system (Guzun, Timohhina et al., 2009).

Figure 9. Proposed model for the mechanism of respiration regulation and energy flux control in cardiac cells. Accordingly a supercomplex, named Mitochondrial Interactosome, that consists of ATP-synthasome-MtCK-VDAC-tubulin couples oxidative phosphorylation with PCr generation and represent a key site of respiration and energy flux control within mitochondria.

Reconstititution experiments with isolated mitochondria and tubulin revealed that tubulin addition restores low affinity of mitochondrial respiration for ADP up to a level of that in permeabilized cardiac cells. These results allow to propose that in cardiac cells mechanism of mitochondrial respiration regulation and control of energy fluxes involves a complex of ATP-synthetase, respiratory enzymes, ANT, MtCK, VDAC and tubulin, named Mitochondrial Interactosome, that couples oxidative phosphorylation with MtCK reaction and facilitates intramitochondrial ATP/ADP cycling (Figure 9). Putative existence of Mitochondrial Interactosome in cardiac cells conforms to the fundamental theory of Peter Mitchell about vectorial metabolism and supports the theories of intracellular energy transport by phosphotranfer networks (Mitchell, 1979; Wallimann, Wyss et al., 1992; Wyss and Kaddurah-Daouk, 2000; Dzeja and Terzic, 2003; Schlattner et al., 2006; Vendelin et al., 2007; Saks, Kaambre et al., 2008). The existence of similar supercomplex, i.e. ATP-synthasome, VDAC and HKII, have been previously identified in cancer cells (Pedersen, 2008)
2. Mitochondria-cytoskeleton interaction: Distribution of β-tubulins in cardiomyocytes and HL-1 cells (Article II)

As evidenced above (Article I), the majority of ATP synthesized in oxidative phosphorylation is exploited in MtCK reaction for the synthesis of PCr. According to previous kinetic studies, the efficiency of coupling between MtCK and oxidative phosphorylation relies on limited MOM permeability (Appaix, Kuznetsov et al., 2003; Guzun, Timohhina et al., 2009) and is regulated likely by tubulin (Carre, Andre et al., 2002; Appaix, Kuznetsov et al., 2003; Monge, Beraud et al., 2008; Rostovtseva, Sheldon et al., 2008). In mammalian, several distinct α- and β-tubulin isoforms are present (Cleveland, 1987). Whether particular isoform is involved in regulation of MOM permeability in cardiac cells is not yet known. To address this question, we examined in this study the intracellular distribution and expression of four, wide-spread β-tubulin isoforms (βI, βII (A and B), βIII, βIV) in cardiac and mouse atrial cancerous NB (non-beating) HL-1 cells. Unlike cardiac cells, NB HL-1 cells have substantially higher mitochondrial respiratory affinity for ADP (\(\text{app}K_m=10-20 \, \mu\text{M}\)), suggesting that permeability restrictions at MOM for adenine nucleotides are absent there, and lack the expression of MtCK (Anmann, Guzun et al., 2006; Eimre et al., 2008; Monge, Beraud et al., 2008).

First, the expression of β-tubulin isoforms was examined in protein extracts of rat heart left ventricle and NB HL-1 cells by Western blot analysis. Brain tissue samples and a mixture of tubulins purified from brain were used as a reference to verify antibody reactivity. Western blot analysis revealed that all studied β-tubulin isotypes are present in heart tissue, as well as in brain, whereas in NB HL-1 cells, the expression of βII-tubulin is missing (Figure 10 A). A similar tissue specific expression pattern was found also for MtCK, which was abundant in heart and absent from NB HL-1 cells.

Next, the subcellular distribution of β-tubulin isoforms was assessed immunocytochemically by confocal microscopy. Therefore, cardiac and NB-HL-1 cells were fixed and immunolabelled for β-tubulin isoform specific antibodies. In agreement with Western blot results, all β-tubulin isoforms were visible in cardiac cells, while in NB HL-1 cells, only weak fluorescence signal was seen for βII-tubulin. Furthermore, each β-tubulin isoforms displayed a unique subcellular localization pattern. For example, in cardiac cells βIII-tubulin formed thin transversely aligned streches, that co-localized with the α-actinin, a protein present at Z-lines. Most interestingly, βII-tubulin was found to form chess-board like pattern within the interior of cardiac cell, which was similar to the known localization pattern of mitochondria. Indeed, then βII-tubulin immunolabelled cells were stained with MitoID, their co-localization became apparent (Figure 10 B).
Altogether, these results show that β-tubulin isoforms vary significantly in their expression and distribution pattern between cardiac and NB HL-1 cells and might thus play a different roles in these cells. Tubulin is the main component of mammalian cytoskeleton and apart from its other functions, it has been shown to participate also in regulation of energy metabolism (Monge, Beraud et al., 2008; Timohhina et al., 2009). According to a previously proposed model, tubulin binds to VDAC via its negatively charged C-terminal tail (CTT), that penetrates into positively charged channel lumen and render channel more cation selective (Colombini, 2004; Rostovtseva and Bezrukov, 2012). This assumption is supported by the fact that upon removal of tubulin CTT by subtilisin, its effect on VDAC conductance dissapears (Rostovtseva et al., 2008). The composition of CTT differs significantly across tubulin isoforms and defines their recognition by different cellular factors (Luduena, 1998). It is conceivable thus, that due to their distinct CTT composition, isoforms have different ability to interact with VDAC, and particular isoforms, such as βII-tubulin, might be preferentially engaged in mitochondria functional regulation, as observed in this study.

Interestingly, βII-tubulin expression was identified only in cardiac cells, where MOM permeability to ADP is restricted, while was missing from NB HL-1 cells, where permeability restrictions for ADP are low. According to a MI

Figure 10. A Western Blot analysis of βII-tubulin in rat heart left ventricle and in HL-1 cells. Numbers above the images show the amount of added total protein in µg. B Confocal micrographs of cardiac cell immunolabelled for βII-tubulin and stained with mitochondria specific dye (Mito-ID™ Red). Scale bar 5µm.
model proposed for cardiac cells (Article I, Figure 9), limited permeability of MOM for adenine nucleotides and functional coupling of MtCK with oxidative phosphorylation forms a fundamental basis for realizing metabolic feedback regulation of mitochondria via creatine phosphotransfer. Ample of data suggest that tubulin interacts with VDAC and together with the results of this study, it can be concluded that βII tubulin is the missing component in MI (Monge, Beraud et al., 2008; Rostovtseva, Sheldon et al., 2008). Apart from the functional significance of this interaction in facilitating the coupled MtCK reaction, the advantage of βII-tubulin-VDAC interaction may also be that it prevents cardiac cells from wasting mitochondrial ATP in glycolysis. In cancer cells with Warburg phenotype, such as NB HL-1, hexokinase II (HKII) is bound to VDAC and mitochondrial ATP is channeled directly to glycolytic network. Conversely, in cardiac cells HKII resides instead in cytosol and do not seem to interact with VDAC (de Cerqueira Cesar and Wilson, 1995; Eimre, Paju et al., 2008). In addition, βII-tubulin- binding to MOM may protect heart from apoptosis (Antonsson, 2004; Dorn, 2009). Possible alteration of mitochondria–tubulin interactions in dilated and ischemic cardiomyopathies may explain why the rate of apoptosis can increase several hundred folds in these diseases (Dorn, 2009). This is one of interesting problems for further study.

In constrast, absence of βII-tubulin from NB HL-1 cells, replaced by HKII, and low expression level of MtCK might contribute to the mechanism of Warburg effect by allowing the direct utilization of mitochondrial ATP for glycolysis. In cancer cells, binding of HKII to VDAC increase its affinity for ATP by ~5-fold, and protects HKII from the inhibition of its byproduct, glucose-6 phosphate (Bustamante and Pedersen, 1980).
3. Role of mitochondria-cytoskeleton interactions in respiration regulation and mitochondrial organization in striated muscles (Article III)

The mechanisms of mitochondrial respiration regulation differ significantly between phenotypically distinct muscle types. In oxidative heart and soleus muscles, the increase in mitochondrial respiration rate is observed at almost stable steady-state cytosolic levels of ATP and PCr, whereas in glycolytic muscles (e.g. m. gastrocnemius), substantial decrease in their intracellular content is observed (Bernier-Valentin and Rousset, 1982). At the same time, the affinity of mitochondrial respiration for ADP displays over manifold lower value in oxidative heart and soleus muscle fibers ($K_m^{ADP}=200-400$ µM) than in glycolytic ones ($K_m^{ADP}=10-20$ µM) (Kuznetsov, Tiivel et al., 1996). Given that intrinsic properties of mitochondria do not vary as much between muscle types to explain discrepancies of that extent (Armstrong and Phelps, 1984; Glancy and Balaban, 2011), the underlying cause of these phenomena has remained somewhat controversial. The aim of this work was to study the mechanisms of mitochondrial respiration regulation in different oxidative and glycolytic rat permeabilized skeletal muscle fibers by using high-resolution oxygraphy. In addition, subcellular distribution and expression of putative VDAC permeability regulating protein, βII-tubulin, was assessed by means of confocal microscopy and Western blotting. Following fiber types were examined in this study: soleus (84% slow-twitch oxidative fiber), gastrocnemius red (GR) (51% slow-twitch oxidative fiber), gastrocnemius white (GW) (92% fast-twitch glycolytic fiber) and EDL (76% fast-twitch glycolytic fiber).

3.1. Kinetics of mitochondrial respiration regulation

First, the affinity of mitochondrial respiration for ADP was determined by stimulating mitochondrial respiration with elevating concentrations of exogenous ADP. In accordance with previous observations (Kuznetsov, Tiivel et al., 1996; Saks, Kaambre et al., 2001), striking differences in the values of apparent $K_m^{ADP}$ of mitochondrial respiration was observed between muscle types. As shown in Table 2, the value of apparent $K_m^{ADP}$ is over 60 fold higher in soleus than in EDL and GW fibers. At the same time, the maximal respiration rate ($V_{O2}^{max}$) is almost similar between soleus, GR and EDL, and only 2-fold lower in GW, which corresponds approximately to the known differences in the cell volume occupied by mitochondria in these muscles (e.g. 5,9% and 2,2% in soleus and gastrocnemius, respectively) (Schaper et al., 1985). When creatine was added to activate MtCK reaction, nearly 6-fold and 2.5-fold decline in apparent $K_m^{ADP}$ was observed in soleus and GR fibers, respectively, while no significant change was seen in GW and EDL (Table 2). Thus, in soleus and to a lesser extent also in GR, creatine has the ability to exert control over mitochondrial respiration and can amplify mitochondrial respiration regulation by ADP via activation of coupled
CK reaction. This is in accordance with Western blot results which showed only minor levels of MtCK in EDL and GW muscles and high expression levels in oxidative muscles (i.e. heart, *soleus*, GR).

Next, the efficiency of creatine mediated feed-back between mitochondria and ATPases was studied by pyruvate kinase (PK)/phosphoenolpyruvate (PEP) test. This protocol is based on the assessment of competition between mitochondria and PK/PEP for endogenous ADP (Gellerich and Saks, 1982) and allows to estimate which energy transfer route (e.g. direct adenine nucleotide channeling, creatine phosphotransfer) is utilized to couple mitochondrial respiration with cytosolic ATPases.

<table>
<thead>
<tr>
<th></th>
<th>$V_0$</th>
<th>$V_{\text{max}}$ (ADP)</th>
<th>$V_{\text{max}}$ (ATP+Cr)</th>
<th>$K_m$ADP</th>
<th>$K_m$ADP (+Cr)</th>
<th>PK-PEP inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmolO$_2$·min$^{-1}$·mg$^{-1}$ dry weight fibers</td>
<td></td>
<td></td>
<td>µM</td>
<td>µM</td>
<td>%</td>
</tr>
<tr>
<td>Soleus</td>
<td>1.55±0.09</td>
<td>10.6±0.4</td>
<td>9.8±0.1</td>
<td>294.2±15.9</td>
<td>66.6±4.7</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>GR</td>
<td>2.10±0.08</td>
<td>9.1±0.4</td>
<td>9.0±0.3</td>
<td>122.0±16.0</td>
<td>49.5±2.8</td>
<td>52.8±5.2</td>
</tr>
<tr>
<td>GW</td>
<td>0.70±0.01</td>
<td>3.8±0.1</td>
<td>5.3±0.27</td>
<td>4.5±1.8</td>
<td>3.6±0.3</td>
<td>48.6±0.2</td>
</tr>
<tr>
<td>EDL</td>
<td>1.10±0.05</td>
<td>9.1±0.5</td>
<td>9.4±0.2</td>
<td>7.4±1.7</td>
<td>5.2±0.6</td>
<td>49.0±3.0</td>
</tr>
</tbody>
</table>

Table 2. Respiratory parameters of permeabilized skeletal muscle fibers. Respiration of permeabilized skeletal muscle fibers was measured in the presence of 5 mM glutamate and 2 mM malate in Mitomed solution at 25°C. Values are means ± SEM.

As shown in Table 2, in presence of PK (20 U/ml)/PEP (5 mM) nearly 50% decline in creatine activated respiration rate is seen in GR, EDL and GW fibers, suggesting that a main part of phosphoryl transfer is realized in these muscles *via* direct ATP/ADP channeling which are diffusing freely between mitochondrial and cytosolic compartments due to high MOM permeability for ADP. In contrast, no observable change is seen in respiration rate under similar conditions in *soleus* fibers, consistent with their markedly higher MtCK expression level and low MOM permeability for ADP. Figure 11 highlights that the extent of PK/PEP induced respiration inhibition correlates in different muscle type with the value of apparent $K_m$ADP of mitochondrial respiration.
3.2. Role of βII-tubulin in regulation of respiration and mitochondrial arrangement in skeletal muscle fibers

In cardiac cells, MOM permeability for adenine nucleotides is regulated likely by βII-tubulin (Artcile II). To explore the possibility that βII-tubulin is responsible for striking differences in the values of $K_m^{ADP}$ of mitochondrial respiration between different skeletal muscle types, βII-tubulin expression and intracellular distribution relative to mitochondria was assessed.

Since tubulin is proposed to participate in mitochondria functional regulation in its unpolymerized state (Rostovtseva, Sheldon et al., 2008), both cold-soluble and insoluble protein extracts of muscles were examined for the presence of βII-tubulin and total β-tubulin by Western blot. As demonstrated in Figure 12, the content of free βII-tubulin is relatively high in heart and soleus muscles, while quite modest or totally absent from GR or EDL, GW muscles, respectively. Interestingly, polymerized βII-tubulin tubulin was detected in minor amounts only in heart. Likewise, the content of total β-tubulin, both in cold-soluble and insoluble fraction, appeared somewhat higher in heart, soleus and GR, than in EDL and GW muscles. Densitometric analysis showed that total β-tubulin dimer/polymer ratio remains the same irrespective of muscle type, suggesting that decrease in free β-tubulin levels, and conceivably also in βII-tubulin, is not related to concomitant increase in their polymerized forms, but is rather a result of overall downregulation of these proteins.
Figure 12. Immunoblot of free and polymerized βII-tubulin and total β-tubulin in soluble and insoluble muscle extracts (35 µg protein per lane). The data shown are representative of 3-4 independent experiments.

Next, subcellular localization of βII-tubulin relative to mitochondria and localization of mitochondria relative to Z-lines was assessed by confocal microscopy. Therefore, fixed muscle fibers were co-immunolabelled with antibodies for VDAC, for visualization of mitochondria, α-actinin, for visualization of Z-lines, and for βII tubulin. As depicted in Figure 13, βII-tubulin is present in heart and soleus as thick segregated bundles, that organize transversely to fiber longitudinal axis, while forms rather thin filaments in GR, EDL and GW muscles. Surprisingly, all studied muscle types display clear co-alignment of mitochondria along βII-tubulin streches (Figure 13). Assessment of mitochondria localization relative to Z-lines revealed that mitochondria organize in heart and soleus at the level of A-band, while reside in GR, EDL and GW, close to Z-lines, in agreement with previous electron microscopy observations made for human skeletal muscles (Ogata and Yamasaki, 1997). Thus, it can be deduced that similar spatial remodelling accompany also βII-tubulin localization in different muscle types. Relatively strong immunolabelling of βII-tubulin in EDL and GW fibers, contradicts with Western blot results which may have two plausible reasons. Either part of the βII-tubulin was still left unextracted during sample preparation, as noted previously for axonal microtubules which are also abundant of βII-tubulin (Tashiro and Komiya, 1989); or βII-tubulin antibody binding site is less accessible under denaturating conditions.

It is well known that microtubules direct mitochondria subcellular organization and are responsible for their enrichment at sites of high energy-demand (Reynolds and Hand, 2004; Anesti and Scorrano, 2006). As muscle types varied in their subcellular localization of mitochondria and βII-tubulin, it seems plausible that βII-tubulin localization contributes to the establishment of compartment specific organization of mitochondria in muscle cells.
Figure 13. Immunofluorescent confocal micrographs of distribution of mitochondria (VDAC), βII-tubulin and Z-lines (α-actinin) in fixed heart and skeletal muscle fibers. Optical slices of fibers are obtained at least 0.5 µm beneath sarcolemma and specimens are all oriented so that the long axis of the fiber is directed longitudinally (slightly diagonal in case of Soleus and GR). Scale bar, 2 µm.

To study if the observed spatial remodelling is related to all β-tubulin isoforms or only with a subset, the organization of total β-tubulin and α-actinin was examined in soleus and GW fibers. Interestingly, total β-tubulin accumulated in soleus entirely at the A-band level, as βII-tubulin, whereas in GW, two sub-populations of β-tubulins were observed. One, at the A-band level and the other one at Z-lines, like βII-tubulin and mitochondria (Figure 13). These data indeed agree that specific subset of β-tubulins, such as βII-tubulin, change their subcellular localization in switch from oxidative to more glycolytic muscle type, and could thereby contribute to similar spatial remodelling of mitochondria.
3.3. Study of energy fluxes in permeabilized skeletal muscle fibers by Metabolic Control Analysis

Finally, metabolic control analysis (MCA) was applied to estimate the energy flux control of respiration regulation in permeabilized soleus and GW fibers. This method, originally derived by (Groen, Wanders et al., 1982) is based on quantification of flux control coefficients (FCC), which are ratios of the fractional change in system variable to fractional change in the biochemical activity that evoked the change. This method allows to estimate the control that a certain reaction exerts on the steady-state flux of a given metabolic pathway.

To determine experimentally the FCC of a reaction, titration with specific enzyme inhibitors was performed. Two different protocols were exploited: 1) direct ADP activation, and 2) creatine activation in presence of MgATP and PK/PEP. With GW muscle fibers, only first protocol was used, due to low stimulatory effect of creatine.

Table 3. Flux control coefficients of MI complexes in soleus and GW. The values of FCC were determined in measurements according to two protocols: a) direct activation with 2 mM ADP and 2) activation with 20 mM creatine activation in presence of MgATP, 20 IU/ml PK and 5 mM PEP system. *Data for cardiomyocytes is taken from Tepp et al. 2011 for comparison (Tepp et al., 2010). Data are represented as average values± SEM for ≥3 experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ADP activation</th>
<th></th>
<th>Creatine activation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux control coefficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soleus</td>
<td>GW</td>
<td>CM 1</td>
<td>Soleus</td>
</tr>
<tr>
<td>ANT CAT</td>
<td>0.61±0.04</td>
<td>0.90±0.05</td>
<td>0.20±0.05</td>
<td>0.86±0.05</td>
</tr>
<tr>
<td>ATP-synthase Oligomycin</td>
<td>0.44±0.03</td>
<td>0.67±0.02</td>
<td>0.065±0.01</td>
<td>0.61±0.07</td>
</tr>
<tr>
<td>Complex I Rotenone</td>
<td>0.69±0.05</td>
<td>0.54±0.06</td>
<td>0.20±0.04</td>
<td>0.71±0.02</td>
</tr>
<tr>
<td>Complex III Antimycin A</td>
<td>0.47±0.01</td>
<td>0.82±0.01</td>
<td>0.41±0.08</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>Complex IV NaCN</td>
<td>0.73±0.03</td>
<td>0.84±0.01</td>
<td>0.39±0.09</td>
<td>0.94±0.01</td>
</tr>
<tr>
<td>MtCK DNFB</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Sum</td>
<td>3.05±0.06</td>
<td>3.77±0.02</td>
<td>1.33±0.31</td>
<td>4.49±0.03</td>
</tr>
</tbody>
</table>

As represented in Table 3, FCCs in soleus are higher in presence of creatine, than with ADP alone. This is in agreement with the data obtained for permeabilized cardiac cells (Tepp, Timohhina et al., 2010) and emphasize that flux control is more efficient in presence of creatine. In both, soleus and GW, the sum of FCCs (4.49±0.03 and 3.77±0.02, respectively) largely exceeds the unity, even with ADP activation alone (3.05±0.06 in soleus).
Taken together, the results of this study show that creatine mediated feedback regulation of mitochondrial respiration is significantly more efficient in soleus than in GR, EDL and GW fibers, and that this efficiency is dependent on the permeability of MOM for ADP. In cardiac cells, restriction at MOM for ADP enhance the affinity of MtCK for creatine and for intramitochondrial MgATP (Guzun, Timohhina et al., 2009), indicating that permeability restrictions at MOM serve to facilitate feed-back via creatine. In addition, limited permeability of MOM for ADP has been shown to enhance intramitochondrial ATP/ADP cycling in cardiac cells (Jacobus and Saks, 1982; Saks et al., 1995; Saks et al., 2008; Guzun, Timohhina et al., 2009), explaining why endogenous ADP is inaccessible to PK/PEP in soleus, but is readily accessible in GR, EDL and GW fibers. These data allow to propose that highly oxidative muscles, such as heart and soleus, achieve metabolic homeostasis through limiting the permeability of MOM for ADP and by upregulating the expression of MtCK, that together promote intramitochondrial ATP/ADP cycling and rapid replenishment of cytocolic PCr reserves via coupled CK reaction in mitochondria.

Interestingly, βII-tubulin expression level increased proportionally with muscle oxidative capacity (heart≥soleus>GR>EDL>GW) and with a decrease in MOM permeability for ADP. This finding is in accordance with earlier observations made for NB HL-1 cells (Article II), where βII-tubulin expression was absent concomitant with over manifold higher apparent K\textsubscript{m}
\textsc{ADP}. Other studies have reported augmented expression of βII-tubulin in brain (Narishige, Blade et al., 1999; Nakamura, Yamamoto et al., 2003; Leandro-Garcia, Leskela et al., 2010; Guzun et al., 2011), where respiratory affinity for ADP is also rather low (K\textsubscript{m}
\textsc{ADP} 100 µM) (Monge, Beraud et al., 2008). Finally, evidence exist that both βI-tubulin and βII-tubulin isoforms (Narishige, Blade et al., 1999) are upregulated during cardiac hypertrophy, a phenomenon accompanied among other factors, also by depression of MOM permeability for ADP (Das, Wong et al., 2008). Thus, βII-tubulin appears to be an important regulatory factor for finetuning creatine mediated feedback control of mitochondrial ATP-production in distinct muscle and cell types.

Given that mitochondria co-aligned along βII-tubulin in distinct muscle types, it seems likely that additional cellular factors regulate the binding of tubulin to VDAC. One of the possibility could be that VDAC phosphorylation state determines its interaction with tubulin as noted previously in vitro (Sheldon, Maldonado et al., 2011). Whether this mechanisms underlies energy flux control through MOM under different cellular context or metabolic requirements remains to be tested in future.

In addition, our results revealed that βII-tubulin belongs to a subset of β-tubulins which subcellular localization relative to Z-lines differs between muscle types concomitant with similar remodelling of mitochondria, suggesting that βII-tubulin could be an important player in compartment specific organization of mitochondria. In oxidative muscles, precise organization of mitochondria relative to myofibrils is a necessary prerequisite for establishing tightly coupled
complexes between mitochondria and ATPases, i.e. ICEU-s (Saks, Kaambre et al., 2001; Saks et al., 2010; Saks et al., 2011). Then mitochondria in cardiac cells are disorganized by trypsin treatment, the value of \( K_m^{ADP} \) decrease over manifold (Appaix, Kuznetsov et al., 2003; MacAskill and Kittler, 2010) and the fidelity of mitochondrial and ATPase cross-talk via creatine phosphotranfer becomes less efficient (Guzun, Timohhina et al., 2009). Thus, the absence of PK/PEP effect in soleus in presence of creatine, may partly be also due to their more strategically localized mitochondria relative to myofibrillar ATPases, that in turn could be aided by βII-tubulin subcellular localization.

Finally, MCA revealed that the sum of FCCs largely exceeds the unity in both soleus and GW muscles, in conditions of either ADP activation alone or in presence of MgATP and creatine. According to Lenaz et al., when FCC exceeds the unity, the pathway components are organized into supramolecular complexes (Lenaz et al., 2010). The advantage of these supramolecular assemblies is direct electron channeling between complexes resulting in increase of oxidative phosphorylation efficiency, prevention of excess oxygen radical formation and stabilization of individual complexes (Schagger et al., 2004; Schafer et al., 2007; Genova et al., 2008). Blue native gel electrophoresis for bovine heart mitochondria extract revealed organization of complex I and III into supramolecular complex (Schagger and Pfeiffer, 2001). Thus, based on these results it appears, that both in soleus and GW fibers respiratory complexes, ANT and, in case of soleus, also MtCK, form a supramolecular assembly within mitochondria.
4. Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactosome (Article IV)

The aim of this review is to describe current understanding of the integrated energy metabolism and metabolic feedback regulation of mitochondrial respiration in cardiac and skeletal muscle cells. The importance of intracellular organization of mitochondria, and the role of structural and functional interactions in facilitating the energetic communication between mitochondria and cytosolic ATPases, is thoroughly dissected. Finally, the knowledges gathered via systemic analysis of integrated energy metabolism is exploited to explain pathogenetic mechanisms of diseases such as cancer and heart insufficiency.

Mitochondria in cardiomyocytes are ordered in highly regular fashion between the arrays of myofibrils and their position is determined by their interaction with various cytoskeletal protein (Kaasik et al., 2001; Seppet et al., 2001; Andrienko et al., 2003; Appaix et al., 2003; Saks et al., 2011). Ample of data suggest that precise localization of mitochondria is crucial in metabolic feedback regulation of mitochondrial respiration (Milner, Mavroidis et al., 2000; Saks, Kaambre et al., 2001; Seppet, Kaambre et al., 2001; Andrienko, Kuznetsov et al., 2003; Appaix, Kuznetsov et al., 2003; Anmann, Guzun et al., 2006). Unlike cardiac cells, NB-HL-1 (mouse atrial tumour lineage) have heterogenous and highly immobile mitochondria network and it is believed, that this underlies their significantly different functional parameters of mitochondrial respiration regulation (Pelloux et al., 2006; Beraud, Pelloux et al., 2009). Of note, the affinity of mitochondrial respiration for ADP is almost 20 times higher in NB HL-1 cells ($K_m^{ADP} 25±4 \mu M$) compared to cardiac cells (Anmann, Guzun et al., 2006).

Biochemical and in vivo $^{31}$P NMR spectroscopy methods have demonstrated that heart maintains remarkable stability of its energy metabolites (ATP, PCr and creatine) over large variety of work-loads – a phenomenon known as metabolic homeostasis of heart (Neely, Denton et al., 1972; Balaban et al., 1986). To explain this phenomenon, three theories have been proposed: equilibrium reaction of cytosolic and mitochondrial CK (Jeneson et al., 2000; Beard et al., 2005; van Beek, 2007; Beard and Kushmerick, 2009; Jeneson et al., 2009), regulation of mitochondria respiration by Ca$^{2+}$ (Balaban, 2002; Dedkova and Blatter, 2008; Korzeniewski et al., 2008; Balaban, 2009), and existence of specific structural and functional interactions between mitochondria and cytosolic ATPases, that result in the emergence of new system level properties (Andrienko, Kuznetsov et al., 2003; Appaix, Kuznetsov et al., 2003; Saks et al., 2006; Saks et al., 2009). Systems Biology approach, which main principle is to study the functionalities that emerge as a result of interactions between system components, has given considerable clarity to this matter, through the application
of permeabilized cell technique, in combination with kinetic analysis, mathematical modeling, whole cell and organ studies (Saks et al., 2009).

Kinetic studies have shown that the affinity of mitochondrial respiration for ADP differs over manifold between permeabilized cardiac cells in situ and isolated mitochondria in vitro (Kummel, 1988; Saks, Vasil'eva et al., 1993). Detailed studies have led to a conclusion that these differences stem from the interaction of mitochondria with cytoskeletal proteins, that restrict the diffusion of ADP to mitochondria through VDAC (Saks, Khuchua et al., 1994; Andrienko, Kuznetsov et al., 2003; Appaix, Kuznetsov et al., 2003; Guzun, Timohhina et al., 2009). The fact that high apparent $K_m^{ADP}$ exist also in synaptosomes (Monge, Beraud et al., 2008), while is over magnitude lower is glycolytic muscle cells (Kuznetsov, Tiivel et al., 1996), largely overrules the possibility that diffusion distance plays a significant role in this matter.

Regulation of MOM permeability for adenine nucleotides is a necessary prerequisite for the functioning of MtCK. Creatine significantly decrease the apparent $K_m^{ADP}$ of mitochondrial respiration in cardiac cells, due the rapid regeneration of ADP in MtCK reaction within the intermembrane space (Saks, Belikova et al., 1995; Saks et al., 2001). Application of ADP trapping system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) in respiratory measurements, have shown that limited permeability of MOM for ADP enhance the coupling of MtCK reaction with oxidative phosphorylation and prevents thereby the accessibility of endogeneous ADP to competitive ADP utilizing systems (Guzun, Timohhina et al., 2009).

Several lines of evidence indicate that tubulin participates in the regulation of MOM permeability. Most importantly, tubulin co-immunoprecipitates with mitochondrial protein VDAC in various normal and cancerous cell lines (Carre, Andre et al., 2002) and electrophysiological experiments with isolated VDAC have shown that only nanomolar concentrations of tubulin are sufficient to induce partial closure of a channel (Rostovtseva, Sheldon et al., 2008). Kinetic studies on isolated brain (Monge, Beraud et al., 2008) and cardiac mitochondria (Article I) support these findings by demonstrating that addition of 1 µM tubulin decrease the apparent $K_m^{ADP}$ of mitochondria respiration from 10-20 µM up to ~170 µM and ~330 µM, respectively.

The coupling between cytosolic and mitochondrial PCr/creatine cycle is realized in mitochondria by Mitochondrial Interactosome, MI, that comprises of ATP synthasome (a term proposed by Pedersen) (Pedersen et al., 2000; Pedersen, 2007; Pedersen, 2007), ATP synthase, ANT, P$_i$ carrier and MtCK (Jacobus and Lehninger, 1973; Saks, Chernousova et al., 1975; Timohhina et al., 2009). The role of this complex is to ensure continous cycling of adenine nucleotides in mitochondria and to certify efficient coupling between PCr synthesis and oxidative phosphorylation. MI model clarifies the opposite functioning of CK in mitochondria and at myofibrils and allows to explain the metabolic aspects of Frank-Starling law of the heart.
Dysfunctioning of integrated energy metabolism is among the leading causes of many cellular pathologies. For instance, in Warburg type cancer cells (e.g. NB HL-1) the composition of MI is significantly altered, with almost complete absence of MtCK (Eimre, Paju et al., 2008) and with hexokinase II bound to VDAC (Jacobus and Lehninger, 1973; Saks, Chernousova et al., 1975; Pedersen et al., 2000; Pedersen, 2007; Pedersen, 2007; Timohhina et al., 2009). As a result, these cells exhibit high glycolytic activity even in presence of oxygen, a phenomenon known as Warburg effect (Warburg, 1956; Warburg, 1956). Another pathogenic mechanism related to the changes in mitochondria function is the opening of mitochondrial permeability pore, PTP, induced by calcium overload and accelerated by production of ROS (Bernardi and Rasola, 2007; Leung and Halestrap, 2008). Both ROS production and PTP opening can be effectively controlled and inhibited by MtCK reaction, which is shown to control the conformation state of mitochondrial carriers and redox state of respiratory chain (Dolder et al., 2003; Meyer et al., 2006).

Cells with high and fluctuating energy demand rely on CK phosphotranfer system to facilitate energy supply to ATPases and to efficiently coordinate ATP utilization with its production. Malfunctioning of CK system leads to serious pathologies of heart, skeletal muscles and nervous system (Wyss and Kaddurah-Daouk, 2000; Dolder, Walzel et al., 2003; Meyer, Machado et al., 2006; Neubauer, 2007). During myocardial infarction or in heart failure significant decline in PCr content occurs (~70%), while total ATP content remains almost unaltered (Neubauer, 2007; Saks et al., 2007). Irregardless of the latter, contraction cycle shortens and contractile force cease, due to decreased regeneration of ATP in functionally important microcompartments. Such localized ATP regeneration by CK system is critical also during myogenesis, when compartmentalized BB-CK reaction supports actin polymerization and myoblast fusion (O'Connor et al., 2008). Studies with transgenic mice models lacking CK isoenzymes or of enzymes responsible for creatine metabolism and transport, have shown that while cells are viable with dysfunctional CK phosphotranfer system, contraction becomes energetically more costly and maximal out-put of muscle declines (Ingwall, 2004; Ventura-Clapier et al., 2004; ten Hove et al., 2005; Ingwall, 2009).

These studies emphasize the importance of compartmentalized CK reactions in distinct cellular processes and underpin the diagnostic value of creatine kinase system functioning for the prognosis of heart and skeletal muscle diseases. Currently non-invasive $^{31}$P and $^1$H NMR imaging in combination with spectroscopic imaging has been elaborated to assess the PCr/ATP ratio and PCr, creatine and ATP cellular content, and to estimate thereby the energy state of the skeletal muscles in patients (Neubauer, 2007). Further challenge in studies of energy metabolism is to develop bioprobes for imaging of metabolic microdomains of ATP and to identify key proteins responsible for their formation (Weiss et al., 2006; Neubauer, 2007).
CONCLUSIONS

1. In cardiac cells, coupling of MtCK reaction with oxidative phosphorylation is highly efficient - almost all ATP synthesized within mitochondrial matrix is utilized in MtCK reaction for the production of PCr (PCr/O₂=5.68±0.14).
2. Binding of heterodimeric tubulin to MOM underlies selective permeability restrictions for adenine nucleotides in cardiac cells.
3. In cardiac cells, βII-tubulin is bound to MOM and is the missing component of Mitochondrial Interactosome.
4. The efficiency of creatine mediated feedback regulation of mitochondrial respiration regulation is dependent in different muscles on the permeability of MOM for ADP.
5. βII-tubulin expression increase proportionally with muscle oxidative capacity (heart≥soleus>GR>EDL>GW) and with a decrease in MOM permeability for ADP, suggesting that βII-tubulin is an important factor for finetuning the efficiency of creatine mediated feedback regulation of mitochondrial respiration in muscles.
6. βII-tubulin spatial reconfiguration is accompanied by similar remodelling of mitochondria, indicating that βII-tubulin subcellular localization may contribute to the establishment of compartment specific organization of mitochondria in muscle cells.
7. ANT, ATP-synthase, VDAC, respiratory enzymes and MtCK (only in soleus) organize into supercomplex in soleus and GW muscles.
8. Creatine mediated feedback signalling is crucial for cells with high and fluctuating energy demands and malfunctioning of this system leads to serious pathologies of heart, skeletal muscles and nervous system.
REFERENCES


respiration by ADP in permeabilized heart and liver cells. *Biochem Biophys Res Commun*, 208 (3), 919-926.


Scholte, H. R., Weijers, P. J. & Wit-Peeters, E. M. 1973. The localization of mitochondrial creatine kinase, and its use for the determination of the


(miCK) and adenine nucleotide translocase (ANT) coupling. *Biophysical Journal*, 660a-660a.


ABSTRACT

Highly oxidative muscles, such as heart and soleus, rely almost entirely on mitochondrial ATP to support the work of contractile apparatus. It is essential thus, that ATP production rate in mitochondria would correspond to its turnover in cytosol. Yet, the cytosolic concentrations of relevant signal molecules, ATP and PCr, stay almost unaltered in highly oxidative muscles irrespective of muscle work-load. This puzzling discovery has obscured the understanding of how ATP production rate in mitochondria is matched with its turnover in cytosol.

The aim of this thesis was to study the mechanisms of mitochondrial respiration regulation in permeabilized cardiac cells and skeletal muscle fibers and to elucidate the role of tubulin in mitochondria functional regulation. To this end the kinetics of respiration regulation was analyzed by oxygraphy and the role of tubulin was examined by confocal microscopy, oxygraphy and by biochemical tools.

Kinetic analysis of mitochondrial respiration in cardiac cells showed that the majority of ATP synthesized in mitochondrial matrix is exploited for PCr synthesis by MtCK (PCr/O2 ~6), showing thus that coupling between PCr synthesis and oxidative phosphorylation is highly efficient. Previous kinetic studies on cardiac cells have demonstrated, that limited permeability of mitochondrial outer membrane (MOM) for ADP, is crucial for the efficiency of this coupling. To validate if tubulin participates in this regulation, reconstitution experiments with isolated cardiac mitochondria were performed. It was found that 1 µM tubulin decrease mitochondrial respiratory affinity for ADP over 20-fold (apparent $K_m^{ADP}$ increased from 11±2 to 330±47 μM), which was reversed by the addition of creatine. Thus, these data clearly show that tubulin binding to MOM induce selective permeability restrictions for ADP in cardiac cells. Competition assay of mitochondria and PK/PEP for endogneous ADP on skeletal muscles revealed, that similarly to cardiac cells, the prominent feedback signal in highly oxidative soleus muscle is creatine. The efficiency of this feedback was significantly lower (~50% decline in respiration rate upon PK/PEP addition) in glycolytic muscles (EDL, GW), where MOM permeability for ADP was high and MtCK expression relatively modest. Thus, regulation of MOM permeability for ADP appears to be a common mechanisms for muscle cells to finetune the coupling of oxidative phosphorylation with cytosolic ATPases via CK reactions.

Analysis of the distribution of β-tubulin isoforms in cardiac cells, revealed that βII-tubulin participates in this regulation. Its expression was relatively high in oxidative, heart and soleus muscles, and low or entirely missing from muscles (GR, EDL, GW) and cells (NB HL-1), where restrictions at MOM for ADP were absent. Furthermore, it was found that βII-tubulin belongs to a subset of β-tubulin isoforms which subcellular localization is remodelled in parallel with mitochondrial, suggesting that its subcellular localization could direct compartment specific organization of mitochondria in muscle cells. Finally
MCA revealed that the sum of flux control coefficients largely exceed the unity in both *soleus* and GW muscles, suggesting that respiratory enzymes, ANT and VDAC, and in *soleus*, also MtCK, are organized into supramolecular complex that facilitate direct metabolite channeling and the efficiency of respiratory control.

In all, these findings demonstrate that highly oxidative muscles, such as heart and *soleus*, rely on creatine mediated feedback signalling of mitochondrial respiration, which efficiency is determined by βII-tubulin induced permeability restrictions at MOM for ADP. As a result, ATP/ADP remains cycling within mitochondria and cytosolic PCr reserves are efficiently replenished *via* coupled CK reaction and oxidative phosphorylation. This mechanism forms the basis for maintaining metabolic homeostasis in the cytosol and for achieving efficient creatine-dependent control of mitochondrial ATP production.
KÖKKUVÕTE

Kõrgelt oksüdatiivsed lihased, nagu süda ja lest sääremarjalihas (*m. soleus*), toetuvad pea täielikult mitokondriaalselt toodetud ATP-le, et toetada kontraktsooniaparaadi tööd. Selleks on oluline, et ATP tootmise kiirus mitokondris vastaks igal ajahetkel selle kulutamisele tsütosoolis. Samas on teada, et peamiste signaalmolekulide, ATP ja PCr, tasemed püsivad tsütosoolis pea muutumatuna, olennama lihaste töökoormusest, jättes arusaamatuks, kuidas ATP toomist mitokondris reguleeritakse.


Mitokondriaalsete hingamise kineetiline analüüs südamerakkudes näitas, et pea kogu mitokondri toodetud ATP kasutatakse ära fosfokreatiini sünteesiks (PCr/O2 ~6). Seega on kreatiini fosforüleerimine mitokondris äärmiselt efektiivselt sidestatud oksüdeeriva fosforüleerimisega ning kreatiinil põhinev tagasiside on peamine metaboolne signaal, mille kaudu koordineeritakse ATP tarbimise ja tootmise kooskõlastatust mitokondri ja tsütosooli vahel. Varasemad kineetilised mõõtmed südamerakkudega on näidanud, et oksüdatiivseks PCr sünteesiks on oluline mitokondri välismembrani läbitavuses piiramine ADP-le. Selleks, et selgitada, kas tubuliin selles osaleb, viidi läbi rekonstitutsiooni eksperimendid südame isoleeritud mitokondritega. Leiti, et 1 µM tubuliin vähendab mitokondriaalse hingamise afiinilisust ADP-le pea 20-korda (näline $K_m^{ADP}$ tõusis 11±2-st 330±47 µM-ni), töstes selle tasemele, mis on omane permeabiliseeritud südamerakkude ja –fiibritele. Sellest võib järeldada, et südamerakkudes on välismembrani põhiselt piiratud läbitavus ADP-le seotud tubuliiniga. Selleks, et hinnata hingamise regulatsiooni mehhanisme erineva metaboolset fenotüübiga skeletilihiasrakkudes, hinnati endogeene ADP kättesaadavust PK/PEP-le kreatiini juuresolekul. Leiti, et sarnaselt südamerakkudele, on ka kõrgelt oksüdatiivses lest sääremarjalihasfibrites kreatiinil põhinev tagasiside efektiivne ning endogeene ADP täielikult kättesaatavate konkurreerivate tagasisidetest väga efektiivne ning endogeene ADP täielikult kättesaatavale tarbivale süsteemile. Samas oli aga endogeene ADP hölpasasti kättesaadav GR ja glükolüütile, EDL ja GW, lihasfibrites, kus PK/PEP lisamine vähendas hingamise kiirust pea 50%. Seega on tänul kõrgemal MtCK ekspressioonile ja madalamale mitokondri välismembrani läbitavuses ADP suhtes, kreatiinil põhinev tagasiside efektiivne mitokondri ja ATPaside vahel oluliselt efektiivsem oksüdatiivsetes kui glükolüütilistes lihastes. Tubuliini isovormide leviku analüüs südame ja NB HL-1 rakkudes näitas, et mitokondri välismembrani läbitavuse kontrollis osaleb...
tubuliini βII isovorm, mis kolokalseerus mitokondriitega südamerakkudes, kuid puudub täielikult NB HL-1 rakkudest. Samuti oli selle isovormi ekspressioin kõrgem lest sääremarjalihases ja langes proporsionaalselt oksüdatiivselt glükolüütilisema ja suurema mitokondri välismembrani läbitavusega lihase poole liikudes (süda ≥ soleus > GR > EDL > GW). Metaboolse kontrolli analüüs näitas, et nii lest sääremarjalihases kui ka GW lihases, on ATP-süntetaas, hingamisahela ensüümid, ANT ja VDAC organiseeritud supramolekulaarsese kompleksi, mis võimaldab metabolite kanaliseerida ja muuta seeläbi hingamise regulatsioon efektiivsemaks.

AKNOWLEDGEMENTS

This work was carried out at the Bioenergetics lab in National Institute of Chemical Physics and Biophysics in Tallinn, at University of Joseph Fourier in Grenoble and at the Centre for Biology of Integrated Systems, in Tallinn University of Technology (TUT), during 2008-2013.

First of all, I would like to express my sincere gratitude to my supervisors Prof Valdur Saks and Prof Madis Metsis for their support, guidance and inspiration.

I thank Prof Erich Gnaiger and Prof Maris Laan for accepting to be my opponents.

I am very grateful to all of my colleagues in the laboratory of Bioenergetics. I thank Kersti Tepp, Natalja Timohhina and Tiia Anmann, for their contribution to this work, for their wonderful company, and for their encouragement. I thank Tuuli Kääambre and Igor Ševtšuk for their guidance and support. I am thankful to Vladimir Tšekulajev, whose enthusiasm and commitment has been really inspiring. I thank Aleksandr Klepinin for introducing me an exciting world of adenylate kinase shuttle and for interesting scientific discussions. I wish to express my special thanks to Maire Peitel for these wonderful, philosophical discussions, for her assistance in every day work and for her endless faith in me.

I am grateful to all of the people at ISBK. My special thanks goes to Marina Suhorutšenko, Jaak Simm, Esta Kägo and Kaur Jaanson (from neurolab). It was really fun to work with you and to have these thrilling scientific and non-scientific discussions. I am deeply grateful to Anne Kaljas for her patience in listening and discussing with me all my crazy ideas, for her enduring encouragement and belief in me. You are truly wonderful!

I thank Heiti Paves for introducing me a wonderful world of confocal microscopy, Anne Kuusk salu for her help in sequencing and for several important reagents and Ivar Järving for his help in ultracentrifugation. I thank Anu Aaspõllu for her support in final stages of my thesis and Raivo Vilu for helping me in several important matters. I thank my colleagues from Grenoble for their contribution to this work. I thank also my dear friends Laura and Reet - it has been the greatest joy to work with you in Tartu and your scientific views have had enormous impact on me.

I am very grateful also to the Archimedes Foundation, the Dora Program, and the graduate school of Biomedicine and Biotechnology for supporting my participation in so many exciting conferences and courses.

My greatest gratitude goes to my family, my mom, my dad, my sister, Viktoria, and my grandparents. Thank you for supporting me during all these years, for believing in me, for helping me in everyday tasks, for putting up my lack of time and certainly also for your amusing and funny company. I am deeply grateful also to Erki, whose positive thinking, fascinating life philosophy and amusing company was and still is the greatest inspiration to me.
CURRICULUM VITAE

Name Minna Varikmaa
Date of birth 01.25.1985
Citizenship Estonian

Contact information

Address Hommiku 30 Tallinn 10922 Estonia
Phone +372 55687962
Email minna.varikmaa@kbfi.ee

Education

2006-2008 University of Tartu (TU), M.Sc., Bioorganic Chemistry, cum laude.
2003-2006 Tallinn University of Technology (TUT), B.Sc., Applied Chemistry and Biotechnology, cum laude.
2000-2003 Tallinn German Gymnasium

Awards

2007 Estonian Students Fund in USA, Elsa D. and Edgar J. Mathiesen Memorial Scholarship.
2006 Scholarship of Jaan Poska.
2006 Scientific contest of Tallinn University of Technology students, 1st prize.

Supplement courses

05.-06.2012 Visiting Research Student, Laboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, France
Project: Mechanisms of skeletal muscle respiration regulation.
04.-05.2010 Visiting Research Student, Center of Quantimetry, University of Claude Bernard, France.
Project: Techniques of cardiomyocyte in vivo transfection and bioluminescence.
10.2011 FinMIT Summerschool in Molecular Biology: Mitochondria and Cancer, Finland.
09.2010 Viral Vectors – Construction and Gene Expression, Institute of Technology, TU, Estonia.
09.-12.2010 Learning and Teaching in University I, Tallinn University, Estonia.
01.2009 Laboratory Animal Science: C-Category Competence Course, TU, Estonia.

**Professional employment**

2008 - ... National Institute of Chemical Physics and Biophysics; senior engineer

**Research activity**

- Mechanisms of regulation of integrated energy metabolism in muscle cells
- Role of tubulin in muscle bioenergetics

**Conference presentations**


- Poster presentation „Investigation of interactions between mitochondrial creatine kinase and ATP/ADP channel“ in the 36th FEBS Congress, Turin, 2011, poster presentation.


ELULOOKIRJELDUS

Nimi Minna Varikmaa
Sünniaeg 01.25.1985 Tallinn, Eesti
Kodakondsus Eesti

Kontaktandmed

Aadress Hommiku 30, Tallinn
Telefon +372 55687962
E-mail minna.varikmaa@kbfi.ee

Haridus

2009-… Keemia ja geenitehnoloogia eriala doktoriõpe, TTÜ
Matemaatika- ja loodusteaduskond
2006-2008 Tartu Ülikool, M.Sc., Bioorgaaniline keemia, *cum laude*
2003-2006 Tallinna Tehnikaülikool, B.Sc., Rakenduskeemia ja
biotechnoloogia, *cum laude*
2000-2003 Tallinna Saksa Gümnaasium

Stipendiumid

2007 Eesti Üliõpilaste Toetusfondi Elsa ja Edgar Mathieseni
nimeline stipendium
2006 Jaan Poska Stipendium.
2006 Tallinna Tehnikaülikooli tudengite teadustööde
konkurss, 1.koht.

Täiendõpe

05.-06.2012 Külalistudeng, Bioenergeetika labor, Joseph Fourier
Ülikool, Prantsusmaa.
Projekt: *Lihasrakkude hingamise regulatsiooni mehhanismid.*
04-05.2010 Külalistudeng, Kvantmeetria keskus, Claude Bernard´i
Ülikool, Prantsusmaa.
Projekt: *Kardiomüotsüütide in vivo transfeksiooni ja
bioluminesentsi tehnikad*
10. 2011 FinMIT Molekulaarbioloogia Suvekool: Mitokonder ja
Vähk, Soome.
09.-12.2010 Õppimine ja Õpetamine Ülikoolis 1, Tallinna Ülikool, Eesti.
09.2009 Fluoresentsi tehnikate põhimõtted, Madriid, Hispaania.
01.2009 Katselooma teadus: C-kategooria kompetentsi kursus, TÜ, Eesti.

Töökokemus

2008-... Keemilise ja Bioloogilise Füüsika Instituut, vaneminsener

Teadustöö põhisuunad

- Integreeritud energeetilise metabolismi regulatsioonimehhanismid lihasrakkudes.
- Tubuliini roll lihasrakkude energia metabolismis

Ettekanded rahvusvahelistel konverentsidel


- Poster presentation „Investigation of interactions between mitochondrial creatine kinase and ATP/ADP channel“ in the 36th FEBS Congress, Turin, 2011, poster presentation.


LIST OF PUBLICATIONS

Original publications


Review articles


- **Varikmaa, M.;** Guzun, R.; Grichine, A.; Gonzalez-Granillo, M.; Usson, Y.; Boucher, F.; Kaambre, T.; Saks, V. Matters of the heart in


Natalia Timohhina, Rita Guzun, Kersti Tepp, Claire Monge, Minna Varikmaa, Heiki Vija, Peeter Sikk, Tuuli Kaambre, Dan Sackett, Valdur Saks

**Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome**

*J Bioenerg Biomembr*, Volume 41, June 2009, 259–275
Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome

Natalia Timohhina · Rita Guzun · Kersti Tepp · Claire Monge · Minna Varikmaa · Heiki Vija · Peeter Sikk · Tuuli Kaambre · Dan Sackett · Valdur Saks

Received: 16 April 2009 / Accepted: 13 June 2009 / Published online: 14 July 2009 © Springer Science + Business Media, LLC 2009

Abstract The aim of this study was to measure energy fluxes from mitochondria in isolated permeabilized cardiomyocytes. Respiration of permeabilized cardiomyocytes and mitochondrial membrane potential were measured in presence of MgATP, pyruvate kinase - phosphoenolpyruvate and creatine. ATP and phosphocreatine concentrations in medium surrounding cardiomyocytes were determined. While ATP concentration did not change in time, mitochondria effectively produced phosphocreatine (PCr) with PCr/O₂ ratio equal to 5.68 ±0.14. Addition of heterodimeric tubulin to isolated mitochondria was found to increase apparent Km for exogenous ADP from 11 ±2 μM to 330 ±47 μM, but creatine again decreased it to 23 ±6 μM. These results show directly that under physiological conditions the major energy carrier from mitochondria into cytoplasm is PCr, produced by mitochondrial creatine kinase (MtCK), which functional coupling to adenine nucleotide translocase is enhanced by selective limitation of permeability of mitochondrial outer membrane within supercomplex ATP Synthase-MtCK-VDAC-tubulin, Mitochondrial Interactosome.

Keywords Respiration · Cardiomyocytes · Mitochondria · Creatine kinase · Creatine · Phosphocreatine · Tubulin

Introduction

Mitochondrial respiration, coupled to production of ATP and fine regulation of energy fluxes to the sites of ATP utilization are vital for normal cell life. In spite of the fundamental progress of knowledge of mitochondrial bioenergetics (Nicholls and Ferguson 2002), the nature of respiratory control and in more general sense, the mechanisms of regulation of energy fluxes during workload changes in the cardiac and other cells in vivo are still highly debated (Balaban 2009; Beard 2005, 2006; Guzun et al. 2009; Saks et al. 2007a, 2006, 2007c; Van Beek 2007, 2008; Vendelin et al. 2000; Wu et al. 2007, 2008). Intensive studies during several decades have accumulated an abundance of data showing compartmentation of adenine nucleotides and the role of phosphotransfer networks in energy transfer (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). Of major importance are the creatine kinase – phosphocreatine circuit (or shuttle) which includes both mitochondrial creatine kinase (MtCK) functionally coupled to the oxidative phosphorylation via adenine nucleotide translocase (ANT) and MM isomor of creatine kinase coupled to MgATPase reactions in myofibrils and at cellular membranes, and the adenylate kinase shuttle (Dzeja et al. 2007; Dzeja and Terzic 2003; Saks et al. 2008, 2007a, 2006,
and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MCK) is depicted as an octamer, located in the mitochondrial intermembrane space (IMS) and attached to the inner membrane surface. In our experiments MCK is activated by creatine (Cr) in the presence of ATP. The final products of MCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The MOM is less permeable than in isolated mitochondria, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. Thus the oxidative phosphorylation is controlled by endogenous ADP produced by the MCK, MMCK and ATPase reactions. The permeabilized cardiomyocytes were supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MCK.

Isolation of mitochondria from cardiac muscle

Mitochondria were isolated from adult rat hearts as described by Saks et al. 1975.

Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously Saks et al. 1991. Wistar male rats (300–350 g) were anaesthetized with pentobarbital and de-coagulated using 500 U of heparin. The heart was quickly excised preserving a part of aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM NaHCO3, 1.5 mM KH2PO4, 1.7 mM MgCl2, 11.7 mM glucose, 10 mM creatine, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM HEPES, pH 7.1. The excised rat heart was cannulated by aorta and suspended in Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 mL/min. The collagenase treatment was performed by switching the perfusion to circulating isolation medium supplemented with 0.03 mg/ml liberase Blendzyme I (Roche) and BSA 2 mg/ml at the flow rate of 5 ml/min for 20–30 min. The end of the digestion was determined

Materials and methods

Experimental protocols

The principles of this study are illustrated by Schemes 1 and 2. Scheme 1 represents isolated mitochondria in vitro when mitochondrial creatine kinase is activated by addition of creatine, and Scheme 2 shows mitochondrion in situ, in permeabilized cardiac cells, surrounded by cytoskeleton proteins (depicted as “X” factor) and myofibrils. The respiratory chain (RC) complexes, ATP synthase (F1F0)
Scheme 1 represents a system related to isolated heart mitochondria, used as a reference system in this work. The respiratory chain (RC) complexes, ATP synthase (F<sub>0</sub>) and Pi carrier PIC are integrated within the mitochondrial inner membrane (MIM). Mitochondrial creatine kinase (MtCK) is depicted as an octamer, located in the mitochondrial inter-membrane space (IMS) and attached to the inner membrane surface. In the experiments MtCK is activated by creatine (Cr) in the presence of ATP. The final products of MtCK-forward reaction are phosphocreatine (PCr) and endogenous ADP. The ADP phosphorylation is visualized by recording the oxygen consumption. In scheme I endogenous intramitochondrial ADP produced by MtCK reaction forms a micro-domain within the intermembrane space. The micro-compartmentalized ADP can either enter into the mitochondrial matrix for phosphorylation or escape into the surrounding medium via voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (MOM).

Scheme 2 represents a mitochondrion in situ, in a permeabilized cardiac cell, surrounded by cytoskeleton proteins (depicted as “X” factor) and myofibrils. The MOM is less permeable than in isolated mitochondria, due to the interactions of VDAC with cytoskeleton proteins. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and inorganic phosphate (Pi). Mitochondrial (MtCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, sarcolemmal) activated by creatine in the presence of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). PEP-PK system removes extramitochondrial ADP produced by intracellular ATP consuming reactions and continuously regenerates extramitochondrial ATP. Endogenous intramitochondrial ADP produced by MtCK forms microcompartments within the IMS and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MtCK.
following the decrease in perfusion pressure measured by a manometer. After the digestion the heart was washed with IM for 2–3 min and transferred into IM containing 20 μM CaCl₂, 10 μM leupeptin, 2 μM STI and 2 mg/ml fatty acid free BSA. The cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded and the pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 μM CaCl₂ and the protease inhibitors. This resuspension-sedimentation cycle with calcium-tolerant cells was performed twice. After that cardiomyocytes were gradually transferred from 20 μM Ca²⁺ IM into free calcium Mitomed (supplemented with protease inhibitors and BSA) and washed 5 times. Each time, slightly turbid supernatant was removed after 4–5 min of the cells’ sedimentation. Isolated cells were resuspended in 1–2 ml of Mitomed solution (Kuznetsov et al. 2008) described below for respiration measurements and stored on ice during measurements. Isolated cardiomyocytes contained 70–90% of rod-like cells when observed under the light microscope.

Permeabilization procedure

In order to study the kinetics of regulation of mitochondrial respiration in cardiomyocytes using different metabolites, the cells sarcollemma was permeabilized by saponin keeping the mitochondrial membranes intact (Kuznetsov et al. 2008; Saks et al. 1998b). The tests for intactness of the outer and inner mitochondrial membranes are described in “Results” section. The permeabilization procedure was carried out directly in an oxygraph chamber with 25μg/mL saponin during 10 min before starting measurements of respiration rates at 25°C and continuous stirring.

Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution (Kuznetsov et al. 2008) containing 0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 3 mM KH₂PO₄, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/mL fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates.

Measurements were carried out at 25°C; solubility of oxygen was taken as 240 nmol/ml (Gnaiger 2001).

In kinetic experiments with different fixed MgATP concentrations, a stock solution of 100 mM MgATP was prepared by mixing equimolar amounts of MgCl₂ and ATP, pH was adjusted to 7.2.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was measured by a spectrofluorimeter (F 2500 DIGILAB, HITACHI, Tokyo, Japan) with a fluorescent cationic dye tetramethylrhodamine methyl ester (TMRM) according to the protocol described earlier (Freedman and Novak 1989). This indicator dye is a lipophilic fluorescent cation which passes cellular and mitochondrial membranes and accumulates within the mitochondrial matrix in a ΔΨm-dependent manner, its fluorescence intensity decreases when the dye is accumulated by mitochondria (Nicholls and Ferguson 2002). The decrease of fluorescence intensity shows mitochondrial energization and is proportional to ΔΨm. This property has been used to dynamically monitor ΔΨm in mitochondria in situ in permeabilized cardiomyocytes. The excitation wavelength was 548 nm and emission wavelength 574 nm. Data are reported as arbitrary fluorescence units (AFUs). TMRM was obtained from FluoroProbes®, Interchim, France, dissolved in DMSO to a concentration of 1 mM. Aliquots of this stock solution were diluted in Mitomed solution described above and used in a final concentration of 0.2μM.

The measurements of changes in ΔΨ in isolated mitochondria and in mitochondria in situ in permeabilized cardiomyocytes induced by substrates, MgATP, PK-PEP system and creatine were performed in the same medium as described above for measurements of oxygen consumption (see above). Shortly, permeabilized cardiomyocytes (or isolated mitochondria) were incubated with 0.2μM TMRM. This stage corresponds to zero polarization of mitochondrial inner membrane or to State 1 of respiration according to Chance (Chance and Williams 1956). Addition of respiratory substrates (5 mM Glutamate and 2 mM Malate) induces polarization of the mitochondrial inner membrane (decrease of AFU due to the accumulation of TMRM inside the matrix). This energy state corresponds to State 2 of respiration according to Chance. The subsequent addition of 2 mM ATP followed by 20 U/mL PK and 5 mM PEP (ADP-trapping system) should induce maximal energization of mitochondria, which corresponds to state 4 respiration. Creatine (10 mM) added in the presence of MgATP and the trapping system for free ADP activates MiCK reaction. Intramitochondrial ADP reduced by activated MiCK is expected to decrease ΔΨm due to its use for ATP synthesis respiration (State 3 according to Chance) and ADP/ATP translocation, both dependent on MiCK-ANT functional coupling. The experiment is terminated by addition of 5 μM uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) which provokes the ΔΨm collapse and thus allows zero level of membrane potential.
Measurements of mitochondrial cytochromes content

For comparative quantitative analysis of the kinetics of regulation of respiration in isolated mitochondria and permeabilized cardiomyocytes, the respiration rates were expressed in nmol of oxygen consumed per minute per nmole of cytochrome aa₃, but not per mg of protein (if not indicated differently). Cytochrome aa₃ content in both cases is representative of the respiratory chain, while proteins contained in cardiomyocytes are not all present in mitochondria. The contents of mitochondrial cytochrome aa₃ in the isolated mitochondria and cardiomyocytes were measured spectrophotometrically according to the method described before (Fuller et al. 1985; Monge et al. 2008). The cells or mitochondria were solubilized with 1% of sodium deoxycholate in phosphate buffer (100 mM KH₂PO₄, pH 8). The differential spectrum (reduced by dithionite versus oxidized cytochromes) was obtained by scanning from 400 to 650 nm using a Cary 50 Bio spectrophotometer (Varian, Palo Alto, USA) or Evolution 600 spectrophotometer (Thermo Electron Scientific Instruments, UK). Figure 1 shows the difference spectrum of cytochromes for isolated mitochondria. The value of peak at 605 nm was used for quantification of respiratory chain cytochrome aa₃ contents (cytochrome c oxidase) both in isolated mitochondria and cardiomyocytes using the extinction coefficient ε value equal to 24 mM⁻¹.cm⁻¹ (Monge et al. 2008; Van Gelder 1966). Protein concentrations were determined using a BCA protein assay kit (Pierce, USA) as a standard.

Isolation and purification of tubulin

Tubulin from rat brain and bovine brain was used with equivalent results. The bovine tubulin was obtained from Cytoskeleton (Boulter, CO, USA). The rat brain tubulin was purified as previously described (Sackett et al. 1991; Wolff et al. 1996). Frozen rat brains were thawed, homogenized in Assembly Buffer (0.1 M MES, 1 mM EGTA, 1 mM MgCl₂, pH 6.9), and centrifuged at 100,000 g. Microtubule protein (tubulin plus microtubule associated proteins) was purified by several rounds of GTP-driven, temperature-dependent polymerization and depolymerization (Sackett et al. 1991). Tubulin was then purified from this material by selective polymerization in high buffer concentration, pelleted by centrifugation, redissolved in Assembly Buffer at 25 mg/ml, and drop frozen in liquid nitrogen (Wolff et al. 1996). In its final form the tubulin used was the αβ-heterodimer (Sackett et al. 1991; Wolff et al. 1996).

Reconstitution studies

Isolated and purified rat heart mitochondria (8 mg/ml) were incubated in Mitomed solution (see above) with 1 μM tubulin for 30 min at room temperature (22°C). After that, the samples were injected into an oxygraph chamber in presence or in absence of 20 mM creatine. Kinetics of activation of respiration were analyzed by successive addition of ADP (0.005–0.01–0.02–0.05–0.1–0.2–0.5–1–2–3 mM). Assay medium additionally contained 0.2% of serum bovine albumin and 1 IU/ml aminopeptidase from potato (Sigma-Aldrich) as an ADP regeneration system. This isoenzyme of aminopeptidase has an exceptionally high ATPase/ADPase ratio (10:1) and can be used for effective regeneration of ADP to maintain steady-state of respiration in the presence of limited amounts of ADP in kinetic studies.

Determination of the rate of PCR production in cardiomyocytes in situ by ion pair HPLC/UPLC

Determination of the rates of PCR synthesis in permeabilized cardiomyocytes in situ under conditions used in respirometry experiments was carried out using ion pair HPLC/UPLC by stopping the reaction typically at 3, 6 and 10 min. 100 μl aliquots of the reaction mixture were withdrawn and added to 200 μl ice-cold 1 M HClO₄ solution, immediately supplemented with 5 μl of 100 mM EDTA and neutralized with 210 μl of 0.952 M KOH in 0.5 – 1 min. The samples were held on ice for additional 10 – 15 min for proper precipitate formation and centrifuged at 16 000 g and 4°C for 2 – 3 min. The supernatants were immediately frozen (-40°C) and analyzed within 5 – 6 h.
Addition of EDTA (final 1 mM) proved to be useful in order to bind traces of Mg²⁺ to suppress any residual enzyme (particularly adenylate kinase, unpublished observations) activity and stabilize the preparations. Separations of CrP, PCh and adenine nucleotides were performed by ultra-performance ion-pair chromatography (UPLC) on a 2.1 × 100 mm ACQUITY UPLC HSS T3 C₁₈ column packed with 1.7 μm particles (Waters) by recording optical density simultaneously at 210 nm and 254 nm for creatine and PCh, and adenine nucleotides, respectively. Sample volumes of 10 μl were injected by autosampler. The mobile phase consisted of buffer A (20 mM KH₂PO₄, 3 mM tetraethylammonium bisulfate (TBAS)) and buffer B (200 mM KH₂PO₄, 10% (v/v) acetonitrile, 0.3 mM TBAS), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2μm membrane filter. The elution was performed at a flow rate of 0.4 ml/min in buffer A for 2 min followed by 1:1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis the column was re-equilibrated by washing for 1 min with water and buffer A for 9 min thus resulting in total time for an analysis 20 min. The column was periodically cleaned by washing with 80% methanol. The retention time for the reagents were defined/checked by measurements with the standard solutions prior to every test series. Stock solutions for calibration (0.1 M) were prepared in 0.2 M KH₂PO₄ at pH 7.0 and stored at −40°C for not more than 2–3 days in order to minimize PCh and ATP degradation. Calibration solutions were prepared in supematant solutions obtained after addition and precipitation of cardiomyocytes as described above.

Results

Figure 2a shows the classical respiratory control analysis. ADP in limited concentrations (0.2 – 0.4 mM) activates respiration but does not maintain the stable value of the rate of oxygen consumption by isolated mitochondria due to its rapid phosphorylation into ATP. As a result the fast transition of respiration from State 3 to the State 4 is observed (Fig. 2a). Addition of 20 mM creatine leads to stabilization of respiratory rate at the level close to the State 3 value (Fig. 2b). In this case a stable level of respiration is maintained by phosphorylation of endogenous ADP produced locally by activated MtCK. In accordance with many earlier data (Jacobus and Lehninger 1973; Meyer et al. 2006; Monge et al. 2008, Saks et al. 2007c, 1975, 2004), these results show that MtCK is able to maintain a maximal rate of respiration by supplying endogenous, locally produced ADP to ANT. Phosphocreatine produced in these coupled reactions leaves mitochondria via VDAC in the outer mitochondrial membrane. It was shown by Gellerich and Saks 1982 that part of ADP locally produced by MtCK in isolated mitochondria is equilibrated between intermembrane space and surrounding medium due to high permeability of the VDAC, but an equal amount of ADP is taken by ANT back to the mitochondrial matrix. This phenomenon can be easily revealed by addition of the ADP trapping system consisting of PK and PEP (Gellerich and Saks 1982; Gellerich et al. 1987, 2002, 2000). When respiration of isolated heart mitochondria was stimulated by creatine in the presence of ATP, addition of PK and PEP decreases the respiration rate to about 50 % of its maximal value. The remnant rate of respiration (up to 50% of VO₂max) was due to the functional coupling between MtCK and ANT with the direct transfer of ADP into the matrix (Vendelin et al. 2004a).

Movement of ADP across the outer membrane of isolated mitochondria can be limited by association of heterodimeric tubulin to VDAC (Monge et al. 2008; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). Under these conditions creatine effectively regulates respiration by increasing the rate of ADP-ATP recycling in the coupled MtCK-ANT system. The fact that tubulin in its dimeric form is able to interact directly with heart mitochondria was confirmed in experiments with isolated heart mitochondria (Monge et al. 2008), by a partial reconstruction of the cytoskeleton surrounding mitochondria. The results shown in Fig. 3 demonstrate that addition of tubulin (1 μM) to isolated heart mitochondria induce an increase in apparent Km for ADP from 11±2 μM to 330±47 μM. The Fig. 3a/b/c show oxygen recordings of the activation of the mitochondrial respiration by exogenous ADP. Figure 3a is control kinetics of respiration regulation of isolated mitochondria. The maximal rate of respiration is observed in the presence of ADP at a concentration of 20 – 50 μM and the apparent Km is very low (11±2 μM), demonstrating the absence of diffusion barriers for ADP into the intermembrane space. Figure 3b shows the increase in diffusion constraints caused by tubulin obviously by direct interaction with VDAC in the outer mitochondrial membrane (Monge et al. 2008; Rostovtseva et al. 2008): respiration rate continues to increase even after addition of ADP at a concentration of 2 mM. In this case, kinetic analysis showed that in the presence of tubulin two populations of mitochondria with different apparent Km appear, one with very high apparent Km for exogenous ADP equal to 330±47 μM (Fig. 3d and e). In the presence of creatine (Fig. 3e) ADP again rapidly activated the respiration and only one population with an apparent Km equal to 23±6 μM was seen due to activation MtCK and increasing the recycling of ADP and ATP in mitochondrial matrix and inner membrane (Kim and Lee 1987; Meyer et al. 2006; Saks et al. 1998a, 1991, 1993). Evidently, creatine easily diffuses into the intermembrane
Fig. 2  a The classical respiratory control—transition between States 2, 3 and 4, according to Chance, in response to addition of limited amounts of ADP (0.2 mM; 0.4 mM) to isolated mitochondria, b Stable State 3 of respiration of isolated mitochondria stimulated by 0.2 mM ADP in the presence of 20 mM creatine. This stability is explained by the continuous production of endogenous ADP by mitochondrial creatine kinase (MtCK) reaction.

The experiments with isolated mitochondria and added tubulin shown in Fig. 3 reproduce well the kinetics of respiration regulation in permeabilized heart cells where the apparent Km for ADP in the control and in the presence of both tubulin and creatine are very close (Fig. 3d and e).

To evaluate quantitatively the relative role of the phosphocreatine flux in energy transfer from mitochondria into cytoplasm in the cardiac cells in vivo, we used the permeabilized cardiac cells in combination with the added, exogenous PK-PEP system to simulate the interaction between mitochondria and glycolytic systems and their competition for extramitochondrial ADP. In permeabilized cardiac cells in situ in the presence of creatine and MgATP (see Scheme 2), MgADP is produced in the MgATPase reactions in myofilbrils and sarcoplasmic reticulum (SR), in the MCK reaction in the mitochondrial intermembrane space and in the MM creatine kinase reaction both in myofilbrils and at SR membranes. If there is direct crosstalk between mitochondria and MgATPases by MgATP supply from mitochondria to ATPases and MgADP back as supposed in several studies (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009), the PCR/O_2 ratio should be significantly less than the theoretically maximal P/O_2 ratio, which is equal to 6 (Nicholls and Ferguson 2002). Thus determination of PCR/O_2 ratio in a system described in Scheme 2 allows us to measure directly the energy fluxes between mitochondria and cytoplasm. Exogenous PK-PEP helps to keep extramitochondrial ADP concentration low and avoid rapid consumption of PCR in the coupled MMCK-MgATPase reactions.

Figure 4a shows a regular quality test for isolated cardiomyocytes used in this work. Addition of ADP in saturating concentration of 2 mM to permeabilized cardiomyocytes induced a State 3 high respiration rate. The respiratory control index usually exceeded 7 (Fig. 4a). Addition of exogenous cytochrome c did not increase the respiration rate, this showing the intactness of the outer mitochondrial membrane, and addition of atracyloside decreased the respiration rate close to the State 2 value, this showing the intactness of the inner mitochondrial membrane (Kuznetsov et al. 2008; Saks et al. 1998b). Only preparations with these characteristics were used in the experiments reported in this work.

Figure 4b shows the behaviour of the study system described by Scheme 2. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to a 2 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 IU/mL) in the
Fig. 3  a Oxygraph recording of the control kinetic of mitochondrial respiration activated by increasing concentrations of exogeneous ADP, b and c Oxygraph recording of the kinetics of mitochondrial respiration regulation in the presence of 1 μM tubulin (3B) and both 1 μM tubulin and 20 mM creatine (3C), d Double reciprocal representation (Lineweaver-Burk) of the kinetic of respiration presence of PEP (5 mM) due to trapping of a significant part of extramitochondrial MgADP. The respiration rate did not return to the State 2 level, this showing that some part of MgADP was channelled back to mitochondria. Subsequent addition of creatine rapidly increased the respiration rate. At a creatine concentration of 10 mM the maximal respiration rate was achieved; therefore, this concentration was used in further experiments. It was shown before that when respiration of mitochondria in permeabilized cardiomyocytes in situ is activated by creatine and MgATP (Guzun et al. 2009) and a high respiration rate is achieved, addition of PK – PEP does not result in a decrease of rate of oxygen consumption. That shows that ADP locally produced by MCK in the intermembrane space is not accessible for exogenous ADP – trapping system, obviously, due to decreased permeability of VDAC as a result of binding of some cytoskeletal protein(s) to this channel. Selective regulation for isolated mitochondria (control, solid circles and straight line), with 1 μM tubulin (triangles and dotted lines) and with either 1 μM tubulin or either 20 mM creatine (empty circles and dashed line), e Comparison of the apparent Kms for exogenous ADP in the presence of tubulin and/or creatine. The values of the Kms indicated above the bars are in μM.

Table 1 summarizes the respiratory parameters of the isolated mitochondria and isolated cardiomyocytes. As it can be seen from Table 1, maximal respiration rates are equal both in isolated mitochondria and cardiomyocytes if calculated per nmol of cytochrome aa3.

Since the mechanism of functioning of ANT is dependent upon and governed by the mitochondrial membrane potential ΔΨ (Klingenberg 2008), it was important to record the changes in ΔΨ under conditions described in Fig. 4b. The results of these measurements are shown in Fig. 5.

Figure 5 shows the recordings of changes in membrane potential in mitochondria in situ in permeabilized cardio-
myocytes. First, cardiomyocytes were permeabilized in fluorimeter cells into which TMRM and PEP were also added. Because of the presence of some endogenous substrates in cardiomyocytes, already some energization of the membrane was observed compared to the zero level at the end of experiments (Fig. 5). This energization was increased (fluorescence decreased) further after addition of glutamate and malate to induce the State 2 respiration. Subsequent addition of ATP did not lead to additional changes in membrane potential: energization of membrane due to the presence of ATP was equilibrated by ADP production in MgATPase reactions. Addition of PK induced transition into a true State 4 respiration and maximal energization of mitochondria due to effective removal of this extramitochondrial ADP. Addition of creatine in a final concentration of 10 mM induced a remarkable decrease in membrane potential and its transition to a new lower steady state level was observed. Addition of an uncoupler CCCP decreased the membrane potential to zero. These experiments show that MtCK in the presence of creatine effectively supplies local ADP to ANT which operates a in so called “productive” exchange mode (ADPin-ATPout) at a high value of ∆Ψ (as compared with the “unproductive” exchange ATPin-ADPout at low membrane potential) (Klingenberg 2008). These data directly show the effective functional coupling between ANT and MtCK.

Very interestingly, measurements of the respiration rates as a function of MgATP concentration at different steps of the experimental protocol described by Scheme 2 and in Figs. 4b and 5 gave remarkable and important results.

Table 1 Basic respiration parameters of isolated rat heart mitochondria and of mitochondria in situ in permeabilized cardiomyocytes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mitochondria in vitro</th>
<th>Mitochondria in situ (permeabilized cardiomyocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V̇o₂, nmolO₂·min⁻¹·mg prot⁻¹</td>
<td>26.37±7.93</td>
<td>7.53±1.61</td>
</tr>
<tr>
<td>V₃₀, nmolO₂·min⁻¹·mg prot⁻¹</td>
<td>187.94±40.68</td>
<td>84.45±13.85</td>
</tr>
<tr>
<td>[Cyt aa₃], nmol·mg prot⁻¹</td>
<td>1.00±0.012</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>V₅₃, nmolO₂·min⁻¹·nmol cyt aa₃⁻¹</td>
<td>187.94±40.68</td>
<td>178.23±33.96</td>
</tr>
<tr>
<td>V₇₃, nmolO₂·min⁻¹·nmol cyt aa₃⁻¹</td>
<td>197.90±31.86</td>
<td>162.63±26.87</td>
</tr>
</tbody>
</table>

* V₀₃ respiration rate in State 2 in the presence of substrates before addition of ADP or ATP
* V₃₀ respiration rate in the presence of 2 mM ADP
* V₇₃,ATP respiration rate in the presence of activated MtCK by 2 mM ATP and 20 mM creatine
Fig. 5 Response of the TMRM fluorescence (excitation 548 nm, emission 574 nm) to mitochondrial respiration changes. Isolated cardiac cells were permeabilized with 25 μg/ml saponin and incubated in MitoTEM solution supplied with 5 mM PEP and 0.2 μM TMRM in a thermostated fluorimeter cell. Addition of the substrates 5 mM glutamate, 2 mM malate caused a decrease in fluorescence indicating the accumulation of TMRM in mitochondrial matrix. 2 mM ATP produced a small change in mitochondrial membrane potential, ΔΨ. Activation of MCK and mitochondrial respiration by addition of 10 mM creatine decreased ΔΨ to a lower steady state level. The uncoupling agent CCCP 5 μM was used to dissipate the membrane potential.

Concerning the role of ATP, ADP, creatine and also selective restriction of diffusion of adenine nucleotides in cardiomyocytes and particularly across the mitochondrial outer membrane in regulation of mitochondrial respiration in vivo. First, these measurements were made in the absence and presence of creatine before addition of PEP-PK system. The MgATP concentrations added in these experiments are shown at the lower abscissa axis in Fig. 6. In the absence of creatine, the respiration rate was increased in response to addition of MgATP due to activation of extramitochondrial MgATPases with the apparent Km equal to 158±40 μM in accordance with many earlier determinations (Saks et al. 2001; Seppet et al. 2001), but the Vmax value was low due to absence of calcium ions in these experiments. In the presence of creatine, the addition of MgATP very rapidly increased the respiration rate to its maximal value and the apparent Km for MgATP decreased to 24±0.8 μM. Under these conditions, MgADP is produced both extramitochondrially in the MgATPase and MM-CrK reactions and in the MCK reaction coupled to ANT (see Scheme 2). To differentiate between these two sources of MgADP, PEP and PK were added. This completely changed the kinetics of respiration regulation: for activation of respiration, addition of much higher concentrations of MgATP was needed; these concentrations are shown on the upper abscissa axis in Fig. 6. In these experiments, the apparent Km for MgATP was increased to 2 mM, in accordance with our recent observations (Guzun et al. 2009). Under these conditions, almost all extramitochondrial ADP is trapped and mitochondrial respiration is exclusively dependent upon ADP supply by MCK (as seen in Fig. 5b), which is only slowly activated by exogenous MgATP most possibly by limited permeability of VDAC in permeabilized cells in situ (Guzun et al. 2009). Thus, for maximal activation of respiration, some extramitochondrial MgADP is needed, and in the presence of creatine rapid recycling of this ADP in the coupled MCK ATP Synthosome system maintains a high respiration rate (Guzun et al. 2009).

Based on the results of experiments shown in Fig. 6, in the next experiments MgATP was used in concentrations of 1, 2 and 5 mM in the presence of creatine (10 mM) and the PEP-PK system for determination of the PCr/O2 ratio in permeabilized cardiomyocytes.

Determination of PCr/O2 ratio was performed with the use of HPLC/UPLC technique for detection and quantification of the compounds of interest, PCR and ATP, in the reaction mixture and with separate measurements of corresponding oxygen uptake with a high-resolution OROBOROS respirometer. Under experimental conditions described above mitochondrial respiration was activated by addition of 10 mM Cr in the presence of fixed MgATP (1, 2, 5 mM) concentration, and extramitochondrial ADP produced by MMCK and ATPase was trapped by the PEP (5 mM) – PK (20 IU/ml) system (Fig. 4b and 5). The product mixture samples were collected at 3, 6 and 10 min.

Fig. 6 Regulation of mitochondrial respiration as a function of the concentration of added exogenous MgATP in the absence (●) and the presence (●) of 20 mM Cr before addition of a PK-PEP system (bottom x-scale) and in the case of supplementation with PEP-PK system (●, top x-scale). In the absence of PK-PEP system, the apparent affinity for exogenous MgATP without Cr (Km(app) = 157.8±40.1 μM), produced by hydrolysis in MgATPase reactions, is diminished due to addition of 20 mM Cr (Km(app) = 24.9±0.8 μM). In the presence of PK-PEP system, apparent affinity for MgATP is significantly decreased (see the text). Maximum rate of respiration in all cases was similar. Addition of ADP-trapping PEP-PK system drastically changes the kinetics of regulation.
after initializing MtCK reaction. Separation and analysis of the mixture of components were performed according to a standard HPLC/UPLC operating procedure (described in Materials and Methods section). The output presents a series of peaks located on the time axis, each corresponding to a compound in the test solution, which passed through UV detectors (Fig. 7a). Estimation of these substances was identified by the authentic samples. The concentration of the components was calculated from the area under corresponding peaks. By plotting peaks matching PCr it could be seen how its amount is increasing in time in the test medium (Fig. 7b).

ATP levels continuously regenerated by the PEP-PK system exhibited no significant change in the trial mixtures (Fig. 8a). However, PCr concentration in the surrounding medium increased rapidly in dependence on MgATP concentration (Fig. 8b).

Since PCr in permeabilized cardiac cells can be produced by different isoforms of creatine kinase (MtCK and non-mitochondrial creatine kinases (myofibrillar, SR and sarcolemmal), to ascertain which amount of PCr content in the samples is of mitochondrial origin, the oxidative phosphorylation was inhibited by 10 μM rotenone (Fig. 8b). The differences of the rates of PCr synthesis in the absence and presence of rotenone at defined MgATP concentrations estimate MtCK contribution in each case (Fig. 8c). Oxygen consumption rises with an increase of MgATP concentration (Table 2) in accordance with kinetic data shown in Fig. 6. The same tendency is observed for the rate of PCr production.

Table 2 summarizes the rates of PCr production and corresponding respiration rates at fixed ATP concentrations. From these data the PCr/O₂ ratio is calculated, the average value is equal to 5.68±0.14, which is close to the theoretical maximal value of 6 (Nicholls and Ferguson 2002).

Discussion

The results of this study show clearly the important role of the ANT - MtCK-VDAC- Tubulin system in regulation of respiration and energy fluxes in the cardiac cells (Fig. 9). ANT in the mitochondrial inner membrane is an integral part of the ATP Synthasome (Chen et al. 2004; Pedersen 2007a, b). Therefore, there seems to be a supercomplex of ATP Synthasome - MtCK – VDAC – Tubulin in contact sites (Brázda 2007) in heart mitochondria which controls the regulation of respiration. This whole complex may be shortly named “Mitochondrial Interactosome,” MI (Fig. 9). This Mitochondrial Interactosome may in some cases

![Chromatograms were obtained by and AGCQUTY UPLC system from permeabilized cardiomyocyte incubations for 5 mM ATP. The samples of the reaction mixture were taken at specified time intervals after the initializing reaction. The positions of PCr and ATP traces on the retention time scale were detected at 2.2 and 8.1 min, respectively. Quantitative assessment of the concentrations of the mixture components was obtained from the peak area.](image-url)

Fig. 7 a Chromatograms were obtained by and AQCDUTY UPLC system from permeabilized cardiomyocyte incubations for 5 mM ATP. The samples of the reaction mixture were taken at specified time intervals after the initializing reaction. The positions of PCr and ATP traces on the retention time scale were detected at 2.2 and 8.1 min, respectively. Quantitative assessment of the concentrations of the mixture components was obtained from the peak area. b Repotted from original chromatograms graph with the peaks corresponding to PCr appearance after 3, 6 and 10 min after activation MtCK reaction by adding 10 mM Cr into the medium.
include also supercomplexes of the respiratory chain (Lenaz and Genova 2007; Vonck and Schafer 2009). Along the cristae membranes the MI contain only MiCK and ATP Synthasome. Direct measurements of energy fluxes from mitochondria into cytoplasm (surrounding medium in experiments with permeabilized cardiac cells) show PCr/O₂ ratios close to the theoretical maximal P/O₂ ratio under conditions similar to those in vivo. These high PCr/O₂ ratios are seen for all three MgATP concentrations up to 5 mM, which is close to physiological concentrations of ATP in cells. Thus, our data clearly show the effectiveness of the transmission of high energy bond from ATP to PCr within the MI. These data leave also little room for direct crosstalk between mitochondria and MgATPases by MgATP and MgADP channeling (Joubert et al. 2008; Kaasik et al. 2001; Kuum et al. 2009) this shows that energy is carried out of mitochondria by PCr fluxes and creatine effectively regulates the MiCK – ATP Synthasome complex due to selective restriction by heterodimeric tubulin of VDAC permeability only for adenine nucleotides but not for creatine or PCr (Guzun et al. 2009). Under these conditions, the amount of ATP and ADP diffusing through MOM is minimal but not zero. Kaasik et al. 2001 and Kuum et al. 2009 made their conclusion of the crosstalk between mitochondria and MgATPases by direct transfer of MgATP and MgADP on the basis of recordings in separate experiments of the amount of calcium in sarcoplasmic reticulum after a rather long period of incubation of permeabilized cells either with ATP, phosphocreatine and ATP, or ATP and respiratory substrates. No reaction rates or energy fluxes were recorded under physiological conditions – activation of MiCK and interaction of mitochondria with other cellular systems, including ATPases, cytoskeleton and the glycolytic system. The absence of such a system analysis does not allow conclusions to be made of the distribution of energy fluxes between mitochondria and cytoplasm in vivo.

In our experiments, both MiCK-controlled respiration and PCr production rates were dependent on MgATP concentration and increased with the elevation of the latter in the interval of 1 – 5 mM (Fig. 6 and 8c). This conforms to our recent kinetic data showing that in permeabilized cardiomyocytes in situ the diffusion of ATP into the intermembrane space is restricted (Guzun et al. 2009). At the same time PCr evidently rather easily diffuses through VDAC into the surrounding medium. Kinetic determina-
Table 2 Measured rates of PCr production, corresponding oxygen consumption and their calculated ratios for fixed ATP concentration in mitochondria in situ

<table>
<thead>
<tr>
<th></th>
<th>( V_{\text{PCr}}^a )</th>
<th>( V_{\text{O}_2}^b )</th>
<th>( V_{\text{PCr}}/V_{\text{O}_2}^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM ATP</td>
<td>0.23±0.02</td>
<td>0.041±0.001</td>
<td>5.80±0.45</td>
</tr>
<tr>
<td>2 mM ATP</td>
<td>0.31±0.02</td>
<td>0.056±0.02</td>
<td>5.44±0.44</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>0.43±0.04</td>
<td>0.074±0.003</td>
<td>5.81±0.48</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.35±0.04</strong></td>
<td><strong>0.061±0.001</strong></td>
<td><strong>5.68±0.14</strong></td>
</tr>
</tbody>
</table>

\( a \) \( V_{\text{PCr}} \) rate of PCr production measured with the use of HPLC/UPLC  
\( b \) \( V_{\text{O}_2} \) rate of oxygen consumption  
\( c \) \( V_{\text{PCr}}/V_{\text{O}_2} \) calculated ratio of PCr/O

Conclusions showed that the affinity of MtCK for exogenous creatine in the permeabilized cardiomyocytes was even increased in comparison with isolated mitochondria, and not changed for phosphocreatine. Because of this rather selective control of VDAC permeability (Rostovtseva et al. 2008, Monge et al. 2008) and functional coupling between PCr and ANT (Saks et al. 2004; Vendelin et al. 2004b), all ATP produced in oxidative phosphorylation is practically completely used for PCr production and ADP is rapidly channeled back through ANT to the mitochondrial matrix.

Our data are in line with an increasing understanding of the importance of the contacts of outer mitochondrial

---

**Fig. 9** Proposed model of regulation of respiration in a supercomplex named Mitochondrial Interactosome, consisting of ATP Synthasome - MtCK-VDAC- Tubulin. Macromolecular ATP synthase is represented as a part of the complex ATP Synthasome (reprinted with kind permission from Peter L. Pedersen, 2007a, b, 2008), with adenine nucleotides carriers (ANC) and phosphate carriers (PIC). Octameric mitochondrial creatine kinase (MtCK) (the structure was kindly supplied by U.Schlatner), located in the mitochondrial intermembrane space (IMS) and attached to mitochondrial inner (MIM) and in the contact sites to outer membranes (MOM). VDAC permeability is selectively regulated by heterodimeric tubulin, which binding to VDAC in intact mitochondrial membrane may be either direct or by some linker proteins (LP). This complex of VDAC with other proteins controls the outcome of adenine nucleotides and PCr fluxes into surrounding medium, and phosphorylation by the ATP Synthasome system is effectively regulated by creatine via MtCK.
membraneVDACwiththecytoskeletonforregulationof
ergyfluxesandmitochondrialrespiratinincardiaccells
(AlievandSaks1997;Anfiuset al.2001;Boudinaet al.
2002;BurelleandHochachka2002;Capetanaki2002;
Colombini2004;Guerreroet al.2005;Kummel1988;
Kuznetsovet al.1989,1996;Lindenet al.1989;Liobikas
et al.2001;RostovtsevaandBezrukov2008;Rostovtseva
1993,1989;Vekslaret al.1995;Vendelin et al.2004b;Zoll
cellsinmanylaboratorieshaveshownanincreased
apparentKmforADPforexogenousADPintheregulation
ofrespirationincomparisonwithisolatedmitochondria
(Anfiuset al.2001;Boudinaet al.2002;Burelleand
Hochachka2002;Guerreroet al.2005;Kummel1988;
Kuznetsovet al.1996;Liobikas et al.2001;Saks et al.
bylocalrestrictionofADPdiffusioninthecellsdueto
bindingofsomecytoskeletalelementscalledfactorXto
the mitochondrial outer membrane(Appaix et al.2003;
Saks et al.1995).Very recently, Rostovtseva et al.
identifiedthisfactorXbyshowingdirectinteractionof
heterodimerictubulinwithVDAC(Rostovtsevaand
Bezrukov2008;Rostovtseva et al.2008).In this work
weshowthatkineticsofregulationofrespirationofisolated
mitochondriawithaddetubulinissimilartothatin
permeabilizedcardiomyocytes.Inbothcasesahigh
apparentKmforexogenousADPisdecreasedwhenMiCK
is activated by creatine (Fig. 3). In intact cells, other
cytoskeletal proteins are also shown to form contacts with
VDAC in the outer mitochondrial membrane, particularly
desmin(Capetanaki2002;Lindenet al.2001)andplecin
(Reipertet al.1999). Rostovtseva et al.2008have directly
shownastronginteractionofpurifiedtubulinwithVDAC
inserted into phospholipid membranes. In the cardiac cells
in vivo, usually only about 30% of tubulin exists in the
polymerized state within the microtubular system, the
remaining part being in the free heterodimeric form
(Tagawa et al.1998). Therefore, the effects observed by
Rostovtseva et al.2008andshowninFig.3maywellbe
valid for in vivo conditions. Interestingly, however, high
apparentKmvaluesarealsocharacteristicforpermeabi-
lized cells (see above) from which dimeric tubulin maybe
thought to leak out. However, that does not happen: in
experiments with use of colchicine (Guerrero2005) to
depolymerizetubulininpermeabilizedcardiomyocytes,
immunolabelingoftubulinbyantibodiesagainstβ
subunits and studies of its localization by confocal
microscopy showed disappearance of the microtubular
network but intensive labeling and diffused intracellular
localization of tubulin, which diffusion may be limited due
to its binding to other cytoskeletal elements and particularly
to the outer mitochondrial membrane (Guerrero2005).
Correspondingly, only a minor decrease of apparent Km for
endogenous ADP was seen (Guerrero2005).

In the intact mitochondrial outer membrane some other
proteins may also be associated with VDAC. These
interactions may result in specific restriction of VDAC
permeability only for adenine nucleotides, but not creatine
or Cr. It has been shown that one of these proteins may be
microtubule-associated protein 2 (MAP2) (Linden and
Karlsson1996;Lindenet al.1989) and cyclic nucleotide
phosphodiesterase (Bifulcoet al.2002). Interestingly,
similar association of tubulin via binding to linker proteins
has been shown for the plasma membrane (Wolff2009).
These data allow supposing that in the contact sites
between inner and outer mitochondrial membranes there is
thesupercomplexMIinthecellsinsituwhitetubulinisa
associated either directly or via linker proteins to VDAC,
which is associated with MiCK-ATP Synthasome complex
(Fig. 9). Earlier, Pedersen et al. have shown the existence
of a similar “supercomplex”, i.e., the ATP Synthasome-
VDAC-Hexokinase 2 in cancer cells that helps in explain-
ingthe Warburg effect (Chenet al.2004;Pedersen2007a,
b,2008). Our earlier studies of cancer cells of the cardiac
phenotype - continuously dividing HL–1 cells are consistent
with the explanation proposed by Pedersen. These studies
have shown that in HL–1 cells apparent Km for exogenous
ADP is very low, creatine kinase is downregulated and
creatinenohaseneffectonrespiration(Ammannet al.2006).
On the contrary, in these cells hexokinase activity is
increased manifold and glucose activates respiration via
activation of membrane-bound hexokinase (not seen in
normal cardiomyocytes) (Eimaret al.2008). These results
show that in the HL–1 cells tubulin is replaced by
hexokinase 2 and creatine kinase is absent in the Mitochon-
drial Interactosome. Thus, alterations in the structure
of M1 may contribute in cancerogenesis. Another way to
change the M1 structure is knock-out MiCK by genetic
manipulations resulting in increasing the energy transfer
in the cells via adenylate kinase pathway (Dzeja et al.2007).

The hypothesis of Mitochondrial Interactosome con-
forms to the fundamental theory of Peter Mitchell about
vectorial metabolism (Mitchell1979,2004). According to
this theory, an important consequence of the organization
of the enzymes into multienzyme complexes is vectorial
metabolism and ligand conduction which brings together
“transport and metabolism into one and the same
chemiosmotic molecular level - biochemical process
catalyzed by group-conducting or a conformationally
mobile group-translocating enzyme system” (Mitchell
1979). For enzymes and catalytic carriers that have
spatially separated binding sites for donor and acceptor
(as MgATP and creatine depicted for MiCK in Fig. 9),
group transfer can be considered as vectorial group translo-
cation (Mitchell 1979). This is true also for movement of substrates and products from carrier to enzyme and via VDAC with selective permeability (see Fig. 9). In his latest reviews Peter Mitchell encouraged a wider use of the chemiosmotic principle and the biochemical concept of specific ligand conduction in explaining organization and operation of metabolic and transport processes within the cell (1979). Today this idea receives increased attention and is certainly another important insight of Peter Mitchell to the understanding of cellular energy conversion processes (Dzeja et al. 2007).

All data reported in this work and recently (Guzun et al. 2009) strongly support the theories of intracellular energy transport by phosphotransfer networks (Dzeja et al. 2007, Saks et al. 2008, 2007a, 2006, 2007c, 2004, Schlattner and Wallimann 2004; Schlattner et al. 2006; Vendelin et al. 2004a; Wallimann et al. 1992, 2007; Wyss et al. 1992). They also show that the popular theories of cells as homogenous medium (Barros and Martinez 2007; Meyer et al. 1984; Wu and Beard 2009) are not compatible with experimental data. An extreme case of these theories is an explanation, which from time to time appears in literature, perfect in its naive simplicity and obviously based on observation of electron micrographs of cardiac cells saying that “mitochondria are “wrapped” partially around the myofilaments with certain degrees of variations. This close apposition of mitochondria to the contractile machinery strategically allows mitochondria to deliver ATP more efficiently to the sites where energy demands are high.” (Hom and Sheu 2009). Others support this view, saying that “since the myofibrils generally have small diameters and are surrounded by tense mitochondria, it is possible that CK-facilitated transport does not play a significant role in vivo” (Wu and Beard 2009). This is some kind of “mechanical bioenergetics” when just looking at electron microscopic images is taken to replace careful biochemical research. Electron micrographs give useful information, but not enough. Our present study presents more clear evidence that the regulation of the cells’ metabolism is a system level property dependent on the interactions of many intracellular structures and systems in the cell (Guzun et al. 2009). These interactions within MI, which lead to new, system level properties, occur within micro- and nanometer scales, much smaller than the diameter of myofibrils. From the point of view of Molecular System Bioenergetics (Guzun et al. 2009; Saks et al. 2007b), an important task is to clarify and describe quantitatively the regulatory mechanisms of the tubulin-VDAC-MtCK-ATP Synthasome supercomplex (Fig. 9), in the interaction with all the other metabolic systems in the cell. In this complex, the behaviour of MtCK cannot be described either by simple solution kinetics nor by even a more simple equilibrium equation (Vendelin et al. 2004b).

Acknowledgements This work was supported by grant from Agence Nationale de la Recherche, France, project ANR-07-BLAN-0086-01 and by grants N 7117 and 7823 from Estonian Science Foundation. The authors thank Dr. Tatiana Rostovtseva, Laboratory of Physical and Structural Biology, NICHD, NIH, Bethesda, MD, USA for active and stimulating discussions.

References

Guerrero K (2005) Organisation structural et fonction metabolique des ICEUs dans le muscle cardiaque et squeletique. Universite Y Fourier, Thèse, Grenoble, France
Rita Guzun, Minna Karu-Varikmaa, Marcela Gonzalez-Granillo, Andrey V. Kuznetsov, Lauriane Michel, Cécile Cottet-Rousselle, Merle Saaremäe, Tuuli Kaambre, Madis Metsis, Michael Grimm, Charles Auffray, Valdur Saks

Mitochondria–cytoskeleton interaction: Distribution of β-tubulins in cardiomyocytes and HL-1 cells

*Biochimica et Biophysica Acta*, Volume 1807, February 2011, 458–469
Mitochondria–cytoskeleton interaction: Distribution of β-tubulins in cardiomyocytes and HL-1 cells

Rita Guzun a, Minna Karu-Varikmaa b, Marcela Gonzalez-Granillo a, Andrey V. Kuznetsov c, Lauriane Michel a, Cécile Cottet-Roussel e, Merle Saaremäe d, Tuuli Kaambre b, Madis Metsis b, Michael Grimm c, Charles Auffray a,d, Valdur Saks a,b,∗

a INSERM U884, Laboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, Grenoble, France
b Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia
c Cardiac Surgery Research Laboratory, Department of Heart Surgery, Immanuel Medical University, Imbruck, Austria
d Functional Ceramics and Systems Biology for Health, CNRS Institute of Biological Sciences, Miluzaf, France

A R T I C L E   I N F O

Article History:
Received 30 November 2010
Received in revised form 15 January 2011
Accepted 31 January 2011
Available online 4 February 2011

Keywords:
Cardiomyocytes
Creatine kinase
HL-1 cells
Mitochondrial interactosome
β-tubulin isoforms
Warburg effect

A B S T R A C T

Mitochondria–cytoskeleton interactions were analyzed in adult rat cardiomyocytes and in cancerous nonbeating HL-1 cells of cardiac phenotype. We show that in adult cardiomyocytes βIII-tubulin is associated with mitochondrial outer membrane (MOM). βII-tubulin demonstrates diffused intracellular distribution. βII-tubulin is colocated with Z-lines and βIV-tubulin forms microtubular network. HL-1 cells are characterized by the absence of βII-tubulin, by the presence of bundles of filamentous βIV-tubulin and diffusely distributed βI- and βIII-tubulins. Mitochondrial isoform of creatine kinase (MtCK), highly expressed in cardiomyocytes, is absent in HL-1 cells. Our results show that high apparent Km for exogenous ADP in regulation of respiration and high expression of MtCK both correlate with the expression of βII-tubulin. The absence of βII-tubulin in HL-1 cells is a result of increased apparent affinity of oxidative phosphorylation for exogenous ADP. This observation is consistent with the assumption that the binding of βII-tubulin to mitochondria limits ADP/ATP diffusion through voltage-dependent anion channel of MOM and thus shifts energy transfer via the phosphocreatine pathway. On the other hand, absence of both βII-tubulin and MtCK in HL-1 cells can be associated with their more glycolysis-dependent energy metabolism which is typical for cancer cells (Warburg effect).

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Recent advances in studies of cellular energetics show that the mechanisms of regulation of energy fluxes and respiration in cells in vivo can be understood only in the framework of molecular system bioenergetics, which considers energy metabolism not only as a network of biochemical reactions, but also takes into account the spatial organization and temporal dynamics of intracellular interactions [1–4]. Interactions between cellular components result in appearance of new, system level properties such as macro- and micro-compartmentation of metabolites, metabolic channeling and functional coupling [3–7]. Thus, they give rise to specific mechanisms, such as energy transfer from the mitochondria to the cytoplasm through phosphotransfer networks [3–9].

Among the cytoskeleton structures, one of the most important roles is attributed to the tubulin–microtubular system. Interactions of mitochondria with tubulin have been observed by many authors [14–18]. The most detailed and pioneering study of the structural interactions performed by Sætersdal et al. in 1990 demonstrated the presence of immunogold anti-β-tubulin labeling at the mitochondrial outer membrane (MOM) in cardiomyocytes, as well as in fibers in close apposition to this membrane [18]. For 20 years, this important observation was left almost unnoticed and unexplained. A possible functional role of this mitochondria-associated tubulin was found in extensive studies of the respiration regulation in permeabilized cells (i.e. in situ mitochondria [19]), when it has been shown that the apparent Km for ADP in oxidative muscle cells (cardiomyocytes, skeletal m. soleus) is 20–30 times higher than in isolated mitochondria [8,20–23]. In addition, the high apparent Km for ADP was found to be decreased by addition of creatine to activate MCK [21,22], or by proteolytic treatment [23]. The apparent Km for exogenous ADP is indicative of the availability of ADP for the adenine nucleotide translocase (ANT) in the mitochondrial inner membrane (MIM) and was proposed to be dependent on the permeability of the voltage-dependent anion channel (VDAC) located in the (MOM) [11,12]. The

* Corresponding author at: Laboratory of Bioenergetics, Joseph Fourier University, 2280, Rue de la Plaine, BP53X-38041, Grenoble Cedex 9, France.
E-mail address: Valdur.Saks@ujg.french (V. Saks).

0005-2728/5 – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j/jbbbio.2011.01.010
strong decrease of the apparent $K_m$ for exogenous ADP induced by trypsin pointed to the possible involvement of some cytoskeleton-related protein(s) in the control of the VDAC permeability originally referred to as "factor X" [11,12,24]. Using immunofluorescence confocal microscopy Appai et al. showed that tubulin and plectin are among cytoskeletal proteins sensitive to the proteolytic treatment [24]. The first established candidate for the role of "factor X" proved to be $\alpha_6$ heterodimeric tubulin, which strongly modulated the VDAC conductance upon binding to channel's protein reconstructed into a planar lipid membrane [17]. Reconstitution experiments indicated that the addition of the heterodimeric tubulin to isolated mitochondria strongly increased the apparent $K_m$ for ADP [16].

The results of these experimental studies led to the assumption that oxidative phosphorylation in cardiomyocytes is effectively regulated by the mitochondrial interactosome (MI), a supercomplex consisting of the ATP synthase, mitochondrial creatine kinase (MtCK), VDAC, tubulin controlling VDAC permeability, and possible linker proteins localized in the contact sites of two mitochondrial membranes [8,25,26].

Pedersen et al. have shown the existence of a similar supercomplex in cancer cells containing the ATP synthase-VDAC-Hexokinase 2 [27-29]. In contrast to the highly oxidative phenotype of metabolite characteristic for adult cardiomyocytes, cancer cells have a glycolytic phenotype characterized by the increased lactate acid production even in the presence of sufficient amounts of oxygen to support mitochondrial function [30-32]. This common metabolic hallmark of malignant tumors was discovered by Otto Warburg and is known as the "Warburg effect" [33,34]. Our earlier studies of mouse cancerous HL-1 cells of cardiac phenotype have shown that the apparent $K_m$ for exogenous ADP is very low and creatine has no effect on their respiration [35,36]. These functional properties of HL-1 cells appear related to alterations in the structure of the mitochondrial interactosome [8,32,37].

In the present work, we continue this direction of research by comparative study of the intracellular distribution of different isotypes of tubulin in normal adult cardiomyocytes and HL-1 cells, using confocal fluorescence and immunofluorescence microscopy and Western blotting. We show that the localization and functional role of $\beta$-tubulin isotypes are different in oxidative muscle tissues and HL-1 cells. Most importantly, in adult cardiomyocytes we identified an isotype of tubulin which is associated with mitochondria-$\beta$III-tubulin. The absence of this isotype in cancer cells appears to allow binding of hexokinase 2 to VDAC and to be directly involved in development of the Warburg effect.

2. Materials and methods

2.1. Cell preparation

For this study we used freshly isolated adult rat cardiomyocytes, isolated rat heart mitochondria and non-beating cancerous HL-1 cells of cardiac phenotype developed in Dr. W.C. Claycomb laboratory (Louisiana State University Health Science Center, New Orleans, LA, USA).

2.2. Isolation of adult cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using modified technique described previously [21]. Wistar male rats (300–350 g) were anesthetized with pentobarbital and blood was protected against coagulation by injection of 500 U of heparin. The heart was quickly excised preserving a part of the aorta and placed into isolation medium (IM) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM NaHCO$_3$, 1.5 mM KH$_2$PO$_4$, 1.7 mM MgCl$_2$, 1.17 mM glucose, 10 mM creatine, 20 mM taurine, 10 mM PCr, 2 mM pyruvate and 21 mM HEPES, pH 7.1. The excised rat heart was cannulated by the aorta and suspended in a Langendorf system for perfusion and washed for 5 min with a flow rate of 15–20 ml/min. The collagenase treatment was performed by switching the perfusion to circulating isolation medium supplemented with 0.03 mg/ml collagenase (Roche) and BSA 2 mg/ml at the flow rate of 5 ml/min for 20–30 min. The end of the digestion was determined following the decrease in perfusion pressure measured by a manometer. After the digestion, the heart was washed with IM for 2–3 min and transferred into IM containing 20 $\mu$M CaCl$_2$, 10 $\mu$M leupeptin, 2 $\mu$M soybean trypsin inhibitor (STI) and 2 mg/ml fatty acid free BSA. The cardiomyocytes were then gently dissociated using forceps and pipette suction. Cell suspension was filtered through a crude net to remove tissue remnants and let to settle for 3–4 min at room temperature. After 3–4 min the initial supernatant was discarded, pellet of cardiomyocytes resuspended in 10 ml of IM containing 20 $\mu$M CaCl$_2$ and the protease inhibitors. This resuspension–sedimentation cycle with calcium-tolerant cells was performed twice, after that cardiomyocytes were gradually transferred from 20 $\mu$M Ca$^{2+}$ IM into free calcium Mitomod (supplemented with protease inhibitors and BSA) and washed 5 times. Each time, slightly turbid supernatant was removed after 4–5 min of the cells' sedimentation. Isolated cells were re-suspended in 1–2 ml of Mitomod solution [19] for the labeling with Mitotracker fluorophore or in paraformaldehyde 4 % for fixation.

2.3. Isolation of mitochondria from cardiac muscle

Heart mitochondria were isolated from adult white Wistar rats 300 g body weight, as described by Saks et al. 1975 [58]. The rats were anesthetized with intraperitoneal injection of Pentobarbital (50 mg/kg body weight). Hearts were removed and placed into ice-cold isolation medium containing 300 mM sucrose, 10 mM HEPES, pH 7.2, and 0.2 mM EDTA. Atriums and vessels were cut off and the ventricles finely minced by scissors. After a brief mild homogenization in a glass potter with teflon pestle (clearance 0.7–0.8) at 200 rpm, during 30 sec, tissue underwent proteolytic digestion in the presence of 0.125 mg/ml trypsin for 15 min at 4 °C. The proteolysis was stopped by addition of 0.5 mg/ml soybean trypsin inhibitor (STI). The sample was carefully and briefly homogenized in a glass-tellon homogenizer (clearance 0.7–0.8) at 250 rpm and 4 °C. This homogenization was followed by a second one (300 rpm, 4 °C) using the potter with smaller clearance. After, the homogenate was centrifuged at 1250g for 10 min at 4 °C. The supernatant was carefully separated and centrifuged at 6300g for 10 min at 4 °C. Mitochondrial pellet obtained was re-suspended in 15 ml of ice-cold extraction medium, supplemented with 1 mg/ml fatty acid free bovine serum albumin, BSA, and washed three times applying the principle of differential centrifugation (3800g at 4 °C for 10 min each time), always carefully removing the upper layer of light fraction of damaged mitochondria in the pellet if it was present [38]. The final pellet containing mitochondria was re-suspended in 1 ml of the same isolation medium.

2.4. Cell culture

Cardiac muscle cell line, designated as HL-1 cells, were derived in the Claycomb laboratory from the AT-1 mouse atrial cardiomyocyte lineage [35–37]. Non-beating HL-1 cells (NB HL-1) were obtained from the HL-1 line developed by W. Claycomb by growing them up in different serum (Gibco fetal bovine serum) [35–37]. NB HL-1 cells do not beat spontaneously. These cells maintain cardiac properties characterized by immunolabeling actin, tubulin, desmin, connexin 43, myosin (developmental isoform), dihydropyridine receptors, by the presence of a sodium–calcium exchanger [36,37]. These cells are devoid of sarcomere structures and possess randomly organized filamentous dynamic mitochondria. NB HL-1 possess the electrophysiological characteristics and ionic currents of cardiac cells
HL-1 cells were cultured in fibronectin (12.5 mg/l)–gelatine (0.02%) coated flasks containing Claycomb medium (Sigma) supplemented with 10% foetal bovine serum (PAN Biotech GmbH), 2 mM L-glutamine (PAN Biotech GmbH), 0.1 mM norepinephrine (Sigma), ascorbic acid 0.3 mM (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) in a humid atmosphere of 5% CO2 / 95% air at 37°C. Cells were cultured in Lab-Tek® chambered coverglass, chamber volume 0.5 ml.

2.5. Sample preparation for Western blot analysis

Male Wistar rats (300–350 g) were anesthetized with pentobarbital, de-coagulated using 500 U heparin and decapitated. Approximately 50 mg samples of the brain tissue were quickly removed, weighed in cryovials and frozen in liquid nitrogen. At the same time, heart was quickly excised, ca. 50 mg samples of the left ventricle weighed and frozen. The samples were stored at −80°C for not more than 1 month. For the sample preparation the tissues were crashed in liquid nitrogen, calculated amount (10 µl per mg tissue) of the buffer (10 mM Tris, 1 mM MgCl2, 1 mM EDTA, 0.2 µM STI, 2 µM leupeptin, pH 7.4) added, homogenized at room temperature for 1 h with short shaking (Vortex in every 10–15 min, centrifuged at 15,000g for 4 min and the solid residual discarded. The obtained homogenate was supplemented by one-half (50 µl per 100 µl) of the sample buffer (0.2 M tris, 16% SDS, 1% DTT, 0.04% bromophenol blue, pH 6.8) and one-half of 50% glycerol and incubated at 95°C for 5 min. The HL-1 nonbeating cells were washed three times with 1 ml ice-cold phosphate buffered saline (PBS, 0.1 M KH2PO4, 0.15 M NaCl, pH 7.2) and lysed on ice with the Tris/Trion X-100 buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.4), concentrated on 500 µl Vivaspain (100 000 MWCO PES) columns (Sartorius Stedim Biotech S.A.) up to approximately 100 µl, and supplemented by the sample buffer and treated as described above.

The protein concentration was routinely determined using the Pierce BCA Protein Kit as suggested by the manufacturer. Heating of the samples was performed at 60°C for 30 min.

2.6. Western blotting

Electrophoresis was performed on the Mini Protean II from BioRad on 10% polyacrylamide gels in the Tris–tricine buffer solution developed by Schägger and von Jagow [39] by applying of 0.5 µg (tubulin mixture) up to 300 µg of the tissue protein (tissue and cell lysates) as described in Fig. 1. The gels were fixed in 40% methanol and 5% phosphoric acid and, if required, stained with colloidal Coomassie G-250.

Blotting of the unstained gels was performed on the Trans-Blot SD Semi-Dry Transfer Cell (BioRad) using PVDF membranes (Millipore) according to the manufacturer’s instructions. The blotting buffer contained 48 mM Tris, 39 mM glycine, 1% SDS and 20% methanol. The membranes were blocked for 1 h with the skimmed milk/TBS solution (0.2 M Tris, 1.5 M NaCl, 0.1% Tween-20, 5% skimmed milk, pH 7.5) with gentle shaking and washed three times for 10 min with the same solution (without skimmed milk) and once in TBS solution lacking both the skimmed milk and Tween-20. Primary antibodies were diluted in the skimmed milk/TBS solution. Primary antibodies and used dilutions are shown in Table 1. Secondary antibodies were IgG and HRP-conjugated antibodies. The membranes were exposed using the CL-X Posure film and SuperSignal West Dura Extended Duration substrate (SuperSignal West Dura Stable Peroxide Buffer and SuperSignal West Dura Lumino/Enhancer Solution).

Rabbit α-actinin antibody was obtained from Abcam (Abcam, ab22427). Mouse α-actinin antibody was obtained from Sigma (A7811). VDAC antibody was obtained from the Laboratory of Physics and Structural Biology, National Institute of Child Health and Human Development, National Institute of Health, USA. Purified mixture of tubulin obtained as described below [16] was used as reference. They were kindly supplied by D. Sackett, Laboratory of Integrative and Medical Biophysics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA.

2.7. Immunofluorescence

Freshly isolated cardiomyocytes and cultured cells were fixed in 4% paraformaldehyde at 37°C for 15 min. After rinsing with PBS solution containing 2% BSA (bovine serum albumin) cells were permeabilized with 1% Triton X-100 at 25°C for 30 min. Finally cells were rinsed repeatedly and incubated with primary antibody as described above for immunoblotting using concentrations indicated into the Table 1 (in 2% BSA containing PBS solution). The next day
samples were rinsed and stained for 30 min at room temperature with secondary antibody. Secondary antibodies: CyTM 5-conjugated Affini-Pure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146), goat polyclonal secondary antibody to mouse IgG-FITC (Abcam ab6785), goat anti-rabbit IgG, F(ab)2-RTC (Santa Cruz sc3839) were used respecting concentrations recommended by the providers. For co-staining the sequential protocol was applied. Two primary antibodies (one overnight at 4 °C and another for 2 h at room temperature) and two secondary antibodies were used to stain fixed samples with repeated rinsing procedures between every staining step. When the immunofluorescence was supplemented with mitochondria labeling, the fixed and immunostained cells (with primary and secondary antibodies) were incubated for 30 min at 37 °C in the presence of 2500 fold dilution of Mito-ID™ Red detection reagent (Mito-ID™ Red detection kit Enabling Discovery in Life Science, EnzynMed 51007-500). For sharper image these cells were visualized immediately after labeling.

For the study of mitochondria arrangement in cardiomyocytes and HL-1 cells, freshly isolated or cultured cells were preloaded with mitochondria-specific fluorescent probe 0.2 μM MitoTracker Red™ and Green™ (Molecular Probes, Eugene, OR) for 2 h at 4 °C for cardiomyocytes and 15 min at 37 °C for HL-1 cells. Images were then analyzed using Velocity software (Improvision, France).

2.8. Confocal imaging

The fluorescence images were acquired with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope (Leica, Heidelberg, Germany) equipped with a 63 x water immersion objective (HCX PL APO 63.0× 1.20 W Corr). Laser excitation was 488 nm for FITC and MitoTracker™ Green, 543 nm for Mito-ID™, and 633 nm for Cy 5, MitoTracker™ Red.

2.9. Measurements of oxygen consumption

The rates of oxygen uptake were determined with high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution [25] containing 0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 3 mM KH₂PO₄, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), pH 7.1, 2 mg/ml fatty acid free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates. Respiration was activated by addition of creatine to final concentration of 10 mM in the presence of ATP (2 mM). Maximal respiration rate was measured in the presence of ADP, 2 mM. Measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmol/ml [25].

2.10. Data analysis

The experiments were carried out independently in two different laboratories by using cardiomyocytes isolated from 10 animals. Functional data were expressed as means ± SE.

3. Results

In this study we analyze the distribution and possible functional roles of four isoforms of β-tubulins: βI (gene TUBB or TUBBS), βII (gene TUBB2A and TUBB2B), βIII (gene TUBB3), and βIV (gene TUBB4 and TUBB2C) in adult rat cardiomyocytes and mouse HL-1 cells (the classification of tubulin isoforms is based on recommended official names [http://www.ncbi.nlm.nih.gov/gene/]. Brain tissue samples and a mixture of tubulins purified from brain were used as a reference for each separate Western blot. Purified tubulins were obtained as described before (see Materials and methods) and were earlier used in the reconstitution experiments with isolated mitochondria [16]. Western blot analysis revealed the presence of all studied β-tubulin isoforms in rat left ventricular muscle tissue, as well as in brain and the reference mixture sample; in contrast, βII-tubulin was not detected in HL-1 cells (Fig. 1A). The distribution of the sarcomeric isoform of Mtck follows the same pattern of significant abundance in left ventricular muscle tissue and complete absence in cancer cells (Fig. 1B). This result is consistent with earlier data by Eimre et al. [36] who showed that in non-beating HL-1 cells the creatine kinase is presented by βB isozyme only. These results are in agreement with the observation that creatine maximally activates the respiration only.

![Fluorescent microscopy imaging of mitochondria fluorophores, respectively. A: Mitochondria in cardiomyocytes visualized by fluorescent probe 0.2 μM MitoTracker™ Green shows separated individual organelles arranged in the regular lines. Scale bar 10 μm. B: Mitochondria in HL-1 cells 0.2 μM MitoTracker™ Red shows disorganized filamentous mitochondrial network. Scale bar 6 μm.](image-url)
in permeabilized cardiomyocytes and has no effect on respiration in permeabilized HL-1 cells (Fig. 1C).

The regular arrangement of individual mitochondria in adult cardiomyocytes imaged by MitoTracker green (Fig. 2A) dramatically contrasts with the disorganized thread-like mitochondrial reticulum of continuously dividing cancerous, highly glycolytic HL-1 cells (Mitotracker red, Fig. 2B). The striking differences observed in mitochondrial arrangement can obviously be related to the specific structural organization and mitochondria-cytoskeleton interactions in these cells [40].

**Fig. 3.** Immunofluorescent confocal microscopy imaging of various β-tubulin isotypes in fixed cardiomyocytes. A: Longitudinally, obliquely and diffusely distributed total β-tubulin labeled with anti-β-tubulin antibody and FITC. Scale bar 24 μm. B: Tubulin labeled with anti-βIV-tubulin antibody and Cy5. βIV-tubulin shows polymerised longitudinally and obliquely oriented microtubules. Scale bar 21 μm. C: Diffuse intracellular distribution of tubulins labeled with anti-β-tubulin antibody. Scale bar 10 μm. D: Tubulin labeled with anti-βIII-tubulin antibody and FITC demonstrates clearly distinguishable prevalent arrangement in transversal lines colocalized with sarcomeric Z-lines. Scale bar 6 μm. E: and F: Regularly arranged tubulins labeled with anti-βII-tubulin antibody and secondary antibodies: Cy5 (E) and FITC (F). In both cases, separate fluorescent spots organized in distinct longitudinally oriented parallel lines similar to the mitochondrial arrangement (see Fig. 2A) in cardiomyocytes. Scale bar 14 μm (E) and scale bar 12 μm (F).
In order to further analyze the localization of β-tubulin isotypes, fixed cells were labeled first with primary antibodies against the proteins studied and then with fluorescent secondary antibodies (described in Materials and methods) and visualized by fluorescent confocal microscopy (Fig. 3). In control experiments without specific primary antibodies no binding of secondary fluorochrome antibodies was seen (results not shown). Total β-tubulin forms tortuous microtubular structure with longitudinally and obliquely oriented cross filaments in adult cardiomyocytes (Fig. 3A). The contribution of each individual tubulin isotype to these structures was analyzed by isotype-specific immunolabeling (Fig. 3B–F). The characteristic structure of β-tubulin appears to be mainly formed by polymerization of βIV-tubulin (Fig. 3B), whereas β-tubulin is associated with a rather scattered distribution of fluorescent spots typical of its usual short polymerized fragments (Fig. 3C). βII-tubulin is characteristically arranged in transversal lines demonstrating colocalization with sarcomeric Z-lines (Fig. 3D). This is very similar to earlier observation made using the immunogold labeling technique [18]. Thus both βIV-tubulin and βII-tubulin appear at least partially responsible for the formation of the typical rod-like shape of adult cardiomyocytes.

The most interesting and exciting results of this study are related to the arrangement of βII-tubulin as revealed by differential immunofluorescent labeling (Cy5, Fig. 3E and FITC, Fig. 3F). In both cases, βII-tubulin is very regularly localized in rows along the long axis of the cell in an arrangement which is very similar to that of mitochondria (Fig. 2A). This observation is in a good agreement with the earlier findings of an association of an immunolabeling with mitochondrial membranes using immunogold labeling [18]. Double-staining with anti-βII-tubulin and an anti-VDAC antibody against epitope 102–120, involved in the protein binding site, revealed an incomplete overlap of fluorescence (results not shown). This can be explained by the difficulties in immunolabeling VDAC, most probably due to presence of the bound tubulin resulting in a low accessibility of VDAC in cardiomyocytes.

Importantly, immunofluorescent labeling detects only traces of β-tubulin II in isolated mitochondria (Fig. 4A) in contrast to immunolabeled VDAC which is clearly detected in isolated mitochondria (Fig. 4B). This apparent contradiction can be explained by the fact that all cytoskeleton proteins associated with mitochondria are removed through trypsin proteolysis during their isolation.

By contrast with the results observed in cardiomyocytes, fluorescent immunolabeling of fixed HL-1 cells revealed the absence of βII-tubulin (Fig. 5A), confirming the results of Western blot analyses (Fig. 1). The clear colocalization of βII-tubulin with α-actinin in Z-lines in cardiomyocytes is replaced by a rather diffuse distribution in HL-1 cells (Fig. 5B), which can be explained by the absence of the sarcomere structure in HL-1 cells. It is well known that overexpression of βII-tubulin represents a valuable prognostic factor for the patients with aggressive evolution of ovarian, lung, pancreas, breast cancer and melanoma, and, at the same time, a poor probability of benefiting from the standard first-line platinum/taxane chemotherapy [41,42]. This suggests some interdependence between intracellular βII-tubulin distribution and cancer metastasis. In addition, β-tubulin labeling appears similar in cancerous HL-1 cells as in adult cardiac cells (Fig. 5C). The filamentous, bundle-like arrangement of βIV-tubulin in cancerous HL-1 cells (Fig. 5D) and the absence of a polymerized microtubular network could explain that they acquire a spherical shape in suspension and spread in culture.

The intracellular localization of βII-tubulin and βIII-tubulin isotypes was investigated by direct colocalization with Z-lines and mitochondria (Figs 6 and 7). The Z-lines were labeled by α-actin antibodies (Figs 6A and 7A) for imaging of the sarcomere limits in cardiomyocytes. Labeling α-actinin with red fluorescence (Fig. 6A) and βIII-tubulin with green fluorescence (Fig. 6B) revealed significantly overlapping structures (Fig. 6C), suggesting that the previously observed specific transversal localization of βII-tubulin is due to βIII-tubulin binding close to the Z-lines of the sarcomere.

Co-staining of α-actinin, labeled this time with a green fluorescence antibody (Fig. 7A) and mitochondria labeled by a specific red fluorescence probe Mito-ID™ (Fig. 7B) shows that mitochondria are localized very regularly between Z lines at the level of sarcomeres (see merged image in Fig. 7C). The absence of overlap of α-actinin at Z-lines and mitochondria-specific fluorescence reflects the absence of mitochondrial fusion. βII-tubulin labeling with green fluorescent antibodies shows its very regular localization in rows parallel to the long axis of the cell (Fig. 7D) oriented perpendicularly to Z-lines, an arrangement similar to that of mitochondria (Fig. 7E), and their full colocalization in cardiomyocytes (Fig. 7F). Comparison of the results displayed in Fig. 3E and F, and Fig. 7D and F indicates that all mitochondria are covered by βII-tubulin, which can therefore be used as an excellent natural intrinsic mitochondrial marker in adult cardiomyocytes.

In contrast, double labeling of HL-1 cells with antibodies against βII-tubulin (Fig. 8A) and with Mito-ID™ (Fig. 8B) as well as their overlap (Fig. 8C) clearly demonstrates the complete lack of βII-tubulin in cancerous HL-1 cells.

4. Discussion

Detailed comparative analysis of structure-function relationship in the regulation of energy fluxes in adult cardiomyocytes and cancerous HL-1 cells of cardiac phenotype described in our previous works [8,35–37] and in this study have yielded a wealth of information concerning the role of mitochondria–cytoskeleton interactions in shaping the specific pathway of energy transfer by the creatine kinase network in heart cells, as well as about the intracellular arrangement of mitochondria and complete prevention of their fusion in adult cardiomyocytes. These studies also show alteration of the mitochondrial dynamics, energy transfer pathways and metabolic phenotype in

![Fig. 4](image_url)

**Fig. 4.** Immunofluorescent confocal microscopy imaging of βII-tubulin and VDAC co-immunolabeling in isolated rat heart mitochondria. A: Absence of immunolabeling by anti-βII-tubulin (and Cy5) antibodies in isolated mitochondria. B: Strong immunofluorescent labeling of VDAC in isolated heart mitochondria (the interaction of mitochondria with βII-tubulin is removed by proteolysis with trypsin). C: The overlap of co-immunolabeling βII-tubulin and VDAC. Scale bar 29 μm.
cancerous cells that may help to understand the mechanism of the Warburg effect. In this study we found that different isoforms of tubulin have different intracellular distribution and therefore may play different roles in the control of energy fluxes and mitochondrial respiration in cardiac muscle cells. We have identified in adult cardiomyocytes the isotype of tubulin which is colocalized with mitochondria and is connected to the mitochondrial outer membrane—βIII-tubulin. It is co-expressed with MLC2 and by structural interactions with VDAC and ATP synthase they form the mitochondrial intertransome [8,25,26]. This supercomplex, localized at contact sites of two mitochondrial membranes, is a key structure of a specific pathway of energy transport from mitochondria into the cytoplasm by phosphotransfer networks which effectively supply energy for contraction and ion pumps by local regeneration of ATP pools in myofibrils and at cellular membranes [8,25]. This prevents from wasting mitochondrial ATP in glycolytic reactions in spite of the presence of hexokinase-2 in the cytoplasm. βIII-tubulin binding to the MOM may also protect the heart from apoptosis. On the contrary, in cancer cells of cardiac phenotype structural changes in the mitochondrial intertransome—lack of βIII-tubulin, which is replaced by hexokinase-2, and lack of MLC2 contribute to the mechanism of the Warburg effect by making possible the direct utilization of mitochondrial ATP for increased glucose phosphorylation and lactic acid production under aerobic conditions.

4.1. βIII-tubulin isoforms in oxidative cardiomyocytes and glycolytic cancer HL-1 cells

Tubulin is one of the most representative proteins of cytoskeleton which among its other functions has a direct role in energy metabolism participating in the structuring of intracellular micro-compartments, formation of dissipative metabolic structures [43,44] and thus in the regulation of metabolic fluxes [45–49]. Under in vivo conditions cytoskeletal protein tubulin is highly dynamic and undergoes rapid assembly/disassembly turnover by exchange of subunits. Building block of microtubules is a αβ-tubulin heterodimer [50]. In cardiomyocytes, about 70% of total tubulin is present in the polymerized form as microtubules whereas 30% occurs as non-polymerized cytosolic heterodimeric protein [51–54]. Interestingly, after complete dissociation of microtubular system by colchicine tubulin is still present in permeabilized cardiomyocytes, obviously because of its association with other cellular structures [55]. In higher vertebrates there are eight α-tubulin and seven β-tubulins encoded by different genes [50]. The majority of differences between tubulin isoforms are restricted to the 15 residues of the C-terminal (also called as isotype defining region) which is a main site for various alterations by post-translational modifications (PTMs) including tyrosinylation, acetylation, phosphorylation, polyglutamylation, and polyglycylation. In addition to this, C-terminal end has been identified to be a main target for numerous microtubule-associated proteins (MAPs) [56–58]. Differences in the C-terminal composition of tubulin isoforms determine the nature of PTM and affect their interactions with different cellular factors and the pattern of localization, thus explaining observations of this study. Recently, Rostovtseva et al. have proposed a mechanism of interaction between tubulin and VDAC, according to which the negatively charged carboxy-terminal tail (CTT) of tubulin penetrates into the canal lumen interacting with a positively charged domain of VDAC [17,59]. For our experiments we used anti-βIII-tubulin antibody against the CELEDDEA amino acids of the CTT. The labeling of all mitochondria by this antibody shows that the negatively charged CTT of βIII-tubulin is located on the outer mitochondrial membrane surface, very probably in close contact with positively charged region of the VDAC also located on this surface when it is in the closed conformation [60,61]. This explains also the decreased permeability of this channel for adenine nucleotides, as described below.

Fig. 5. Immunofluorescent confocal microscopy imaging of β-tubulin isoforms in fixed HL-1 cells. A: Tubulins labeled with mouse anti-βII-tubulin antibody and Cy5 are practically absent in HL-1 cells. Scale bar 16 µm. B: Tubulins labeled with anti-βIII-tubulin antibody and C: diffuse distribution of tubulins labeled with anti-βII-tubulin antibody. Scale bar 10 µm. D: Immunofluorescence imaging of polymerized tubulin microtubules labeled with anti-βIV-tubulin antibody and Cy5. The filaments show radial distribution from nucleus to cell periphery, creating also inter-connections between branches. Scale bar 18 µm.

Fig. 6. Co-immunofluorescent labeling of α-actinin and βIII-tubulin in fixed cardiomyocytes. A: Characteristic sarcomeric transversal Z-lines labeled with anti-α-actinin antibody and Cy5. B: Tubulins labeled with anti-βIII-tubulin antibody and FITC. C: The overlap of α-actinin and βIII-tubulin. Both proteins are arranged at Z-lines. Scale bar 18 µm.
In contrast, we did not find βIII-tubulin in cancer HL-1 cells of cardiac phenotype (Fig. 8). This observation matches well with previous findings of Hiser et al. [62]. Authors reported the absence of β-tubulin class II protein in 8 from 12 studied human cancer cell lines.

Mitochondria–cytoskeletal interactions, particularly the connection of βIII-tubulin to the MOM may also prevent in significant degree the association of pro-apoptotic proteins to this membrane [63]. This may explain why apoptosis is rare in normal human hearts [64]. Possible alteration of mitochondria–tubulin interactions in dilated and ischemic cardiomyopathies may explain why the rate of apoptosis can increase several hundred folds in these diseases [64]. This is one of the interesting problems for further study.

The presence of βIII-tubulin at the outer mitochondrial membrane explains the very high value of apparent $K_m$ for free exogenous ADP in adult permeabilized cardiomyocytes which is an order of magnitude higher than in isolated mitochondria (3.70 μM compared to 10–15 μM, respectively) [8,20–25]. Complete kinetic analysis of regulation of respiration by mitochondrial creatine kinase (MCK) reaction in permeabilized cardiomyocytes confirmed the conclusions made in experiments with tubulin binding to VDAC [16,17,59] and uncovered specific restrictions of VDAC permeability by tubulin [25]. These experiments showed significant decrease of the apparent affinity for extramitochondrial ATP of MCK localized on the outer surface of inner mitochondrial membrane in the intermembrane space in vivo in comparison with isolated mitochondria. This appears to be due to the diffusion restrictions created by interactions of VDAC in MOM with the tubulin α6 heterodimer [17,25,26,59]. Direct measurements of energy fluxes in permeabilized cardiomyocytes and in perfused hearts using the $^{16}$O isotope tracer coupled with $^{31}$P-NMR spectrometry showed that the ratio of the rates of PCR production to oxygen consumption (VPCR/VO₂) is close to 6, meaning that almost all energy is carried out of mitochondria by phosphocreatine molecules [26,65]. The results of these experimental studies showed that oxidative phosphorylation in cardiomyocytes is effectively regulated by creatine via MCK in the mitochondrial interactosome (MI) [8,26].

In cancer HL-1 cells the apparent $K_m$ for exogenous ADP was found to be in the range of 8–20 μM, which is similar to that of isolated mitochondria [35]. Moreover, in cancer cells creatine did not induce change of the respiration rates due to the absence of MCK [35,36], but it was increased in cancer HL-1 cells in response to the addition of glucose showing its phosphorylation by hexokinase 2 [36].

Previous observations and results of the present study show that the two events—high apparent $K_m$ for exogenous ADP and expression of MCK correlate with the expression of βIII-tubulin. The absence of

Fig. 7. Immunofluorescent imaging of α-actinin, β-tubulin II proteins and fluorescent staining of mitochondria. A: Green immunofluorescence of proteins labeled with anti-α-actinin antibody and FITC, demonstrating the characteristic sarcomeric transverse Z-line. B: Typical regular arrangement of mitochondria labeled with Mitoc-III™ Red. C: Merge image of A and B. Mitochondria (red) are localized exclusively between Z-lines (green). Scale bar 5 μm. D: Tubulins labeled with anti-βIII-tubulin antibody and FITC. Fluorescent spots are regularly arranged (similarly to mitochondria). E: Mitoc-III™ Red labeled mitochondria in cardiomyocyte. F: The overlap of D and E. βIII-tubulin class II (green) is arranged between Z-lines and fully co-localised with mitochondria. Scale bar 5 μm.

Fig. 8. The immunofluorescence of anti-βIII-tubulin antibody (A) of rabbit source (and FITC) and mitochondria labeled with Mitoc-III™ Red fluorophore (B) in HL-1 cells. Image A and merge image (C) shows the absence βIII-tubulin labeling in HL-1 cells. Scale bar 14 μm.
Fig. 9. Different pathways of intracellular energy transfer from mitochondria to cytoplasm in adult cardiomyocytes and HL-1 cells. A: Besmann-Wallimann-Saks pathway of energy transfer via creatine kinase phosphotransfer network in normal adult cardiomyocytes. Tubulin III isotype is co-expressed with MiCK in Mitochondrial Interactosome and energy is channelled to cytoplasm by PCr which regenerate the local pools of ATP (phosphocreatine shuttle). Figure A represents adult cardiac cell (scheme at the left). Free fatty acids (FFA) are taken up by a family of plasma membrane proteins (FATPs), esterified to acyl-CoA which enters the β-oxidation pathway resulting in acetyl-CoA production. Glucose (GLU) is taken up by glucose transporter-4 (GLUT-4) and via the phosphorylation by hexokinase (HK) (depicted as soluble enzyme) with production of glucose-6-phosphate (G6P), enters the Embden-Meyerhof pathway. Pyruvate produced from glucose oxidation is transformed by the pyruvate dehydrogenase complex (PDH) into acetyl-CoA. G6P inhibits HK decreasing the rate of glycolysis. Acetyl-CoA is further oxidized to CO2 in the tricarboxylic acids (TCA) cycle with the concomitant generation of NADH and FADH2, which are oxidized in the respiratory chain (complexes I, II, III and IV) with final ATP synthesis. These pathways occur under aerobic condition. Under anaerobic conditions, pyruvate can be converted to lactate. The insert shown in right panel illustrates functioning of Mitochondrial Interactosome (MI), a key system in energy transfer from mitochondria to cytoplasm. MI-a supercomplex, formed by ATP synthase, adenine nucleotides translocase (ANT), phosphate carriers (PK), mitochondrial creatine kinase (MiCK), voltage-dependent anion channel (VDAC) and bound cytoskeleton protein tubulin (specifically β-tubulin II selectively controlling VDAC permeability) and some linker proteins (LP), is responsible for recycling of ATP and ADP within mitochondria coupled to direct phosphorylation of creatine (Cr) into phosphocreatine (PCr). PCr is then transferred via cytosolic Cr/PCr shuttle to be used by functionally coupled CK with ATPases (acto-myosin ATPase and ion pumps) for regeneration of local ATP pools. B: Warburg-Pedersen pathway of energy transfer in cancer cells. Structure of mitochondrial interactosome is significantly modified; protein β-tubulin class II is replaced by HK, and the absence of MiCK allows all oxidative ATP to be exported directly from mitochondria. As the result, VDAC-bound HK is protected from the Glucose-6-P product inhibition, and uses mitochondrially produced ATP for phosphorylation of glucose and stimulation of glycolytic lactate production. The Glucose-6-P and glycolytic ATP synthesized during lactate production are used in biosynthetic pathways for cell growth and proliferation.
the βIII-tubulin isotype both in isolated mitochondria and in HL-1 cells results in increase of the apparent affinity of oxidative phosphorylation for free ADP. This observation is consistent with the assumption that the binding of βIII-tubulin to VDAC limits ADP/ATP diffusion through MOM.

The increased restriction of diffusion of adenine nucleotides through the MOM and the presence of MCK functionally coupled to ANT in the MI are the most important mechanisms in the pathway of intracellular energy transport by phosphoryl transfer via the creatine kinase–phosphocreatine (PCR) network [3,8,9,66]. The system of compartmentalized creatine kinase isoenzymes and notably mitochondrial CK (MCK) is the prerequisite condition for the efficient intracellular phosphotransfer [3]. The functioning of MI and its role in the energy transfer from mitochondria into the cytoplasm in cardiomyocytes with respiratory phenotype are represented schematically in Fig. 9A. In these cells, energy is transferred from mitochondria by the phosphotransfer PCR–CK circuit or shuttle. This pathway of energy transfer was in details described in Bessman [67,68], Wallmann [9,66] and Saks [3,8,21–26] laboratories and many others. In order to acknowledge the contribution of these three laboratories in the identification of energy transfer via creatine kinase phosphotransfer network it is named the Bessman–Wallmann–Saks pathway.

Analysis of the compartmentalized energy transfer by mathematical modelling [69] showed, in agreement with experimental data, that not more that 6–10% of ATP formed in oxidative phosphorylation is directly transferred out of mitochondria [70]. This, by maintaining local ATP pools, prevents the wasteful use of mitochondrial ATP for glucose phosphorylation by hexokinase-2 and ensures availability of an energy supply matching the specific cell needs (see Fig. 9A).

4.2. The lack of ßIII-tubulin and increased hexokinase-2 open the way to the Warburg effect

One of possible mechanisms triggering the Warburg effect is overexpression of hexokinase-2 [28,29,32,71–73]. In cancer cells, hexokinase-2 is bound to the VDAC and this interaction enhances its affinity for ATP by ~5-fold, and protects the enzyme from the inhibition by its byproduct glucose-6-phosphate [74]. In HL-1 cells hexokinase-2 is also overexpressed and its activity is increased by a factor of 5 in comparison with that observed in adult cardiomyocytes [36,37]. Thus, according to Pedersen's explanation of the Warburg mechanism, hexokinase-2 bound to VDAC actively phosphorylates glucose using mitochondrially synthesized ATP, redirecting it actively through the glycolytic pathway [28,75].

Hexokinase-2 is also prevalent in cardiac muscle cells [36,37,76]. However, it is only in cancer HL-1 cells which lack βIII-tubulin, that hexokinase-2 binds to VDAC, altering cellular metabolism. Thus, binding of βIII-tubulin to VDAC in normal cells prevents the wasting of mitochondrial ATP for increased lactate production that occurs in cancers cells through its binding of hexokinase-2 made possible by the absence of βIII-tubulin. A comparative study of enzyme profiles in HL-1 cells and cardiomyocytes showed an increased activity of several glycolytic enzymes, particularly hexokinase-2 in HL-1 cells [37]. The decrease or absence of MCK and the downregulation of its mRNA were previously reported in human sarcoma, gastric and colon adenocarcinoma [77], indicating that these cells are unable to retain their intracellular creatine pool in the form of phosphocreatine because of the intrinsic low level of MCK [77].

Thus, the mitochondrial interaction in cancer HL-1 cells of cardiac phenotype is lacking of βIII-tubulin and MCK and significantly differs from that of healthy adult cardiac muscle cells incorporating ATP synthesize, ANT, PIC, VDAC and HK-2 bound to VDAC. The absence of βIII-tubulin and MCK in the MI of HL-1 cells allows hexokinase-2 to bind to VDAC through its N-terminal hydrophobic domain [73]. The functioning of the MI in HL-1 cells is represented schematically in Fig. 9B. This pathway of energy transfer may be called the Warburg-Pedersen pathway to recognize the contribution of these distinguished investigator in its identification. In the absence of MCK, mitochondrial ATP is directly carried out from mitochondria and used by VDAC-bound hexokinase-2 for glucose phosphorylation. Glucose-6P produced in this reaction enters the glycolytic and pentose-phosphate pathways, sustaining cellular growth and proliferation.

In conclusion, the remodelling of MI, namely the lack of βIII-tubulin protein that makes possible hexokinase binding may be considered as the structural basis of the Warburg effect, explaining the switch from the energy transfer supporting the respiratory phenotype in normal cardiac cells to the more glycolytic phenotype of cancerous cells (see Fig. 9A and B). To verify this hypothesis, detailed further studies of distribution of hexokinase and tubulin isoforms in different cancer cells are needed.

Acknowledgments

The authors thank Dan L. Sackett, Laboratory of Integrative and Medical Biophysics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA, for reading this manuscript and for very helpful critical discussion. The authors thank Anu Nutt, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia, for skilful technical assistance. This work was supported by INSERM, France, by Agence Nationale de la Recherche, project ANR-07-BLAN-0086-01 France, by grant No. 7823 from the Estonian Science Foundation, SF010114B008 from Estonia Ministry of Education and Science, by National Council of Science and Technology of Mexico (CONACYT) and by a research grant from the Austrian Science Fund (FWF): [P22880-B20].

References


Minna Varikmaa, Rafaela Bagur, Tuuli Kaamble, Alexei Grichine,
Natalja Timohhina, Kersti Tepp, Igor Shevchuk, Vladimir Chekulayev, Madis
Metsis, François Boucher, Valdur Saks, Andrey V. Kuznetsov, Rita Guzun

**Role of mitochondria-cytoskeleton interactions in respiration regulation and**
**mitochondrial organization in striated muscles**

*Biochimica et Biophysica Acta, 2013, in press.*
Valdur Saks, Rita Guzun, Natalja Timohhina, Kersti Tepp, Minna Varikmaa, Claire Monge, Nathalie Beraud, Tuuli Kaambre, Andrey Kuznetsov, Lumme Kadaja, Margus Eimre, Enn Seppet

Structure–function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactosome

*Biochimica et Biophysica Acta*, Volume 1797, January 2010, 678–697
Review

Structure–function relationships in feedback regulation of energy fluxes in vivo in health and disease: Mitochondrial Interactome

Valdur Saks a,b,*, Rita Guzin a, Natalja Timožhina b, Kersti Tepp b, Minna Varikma a, Claire Monge a, Nathalie Beraud a, Tuuli Kaambre b, Andrey Kuznetsov c, Lumme Kadaja c, Margus Einre d, Enn Seppet d

a Laboratory of Fundamental and Applied Bioenergetics, INSEME U884, Joseph Fourier University, Grenoble, France
b Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia
c Cardiac Surgery Research Laboratory, Department of Cardiac Surgery, Innsbruck Medical University, Austria
d Department of Pathophysiology, Faculty of Medicine, University of Tartu, Tartu, Estonia

ABSTRACT

The aim of this review is to analyze the results of experimental research of mechanisms of regulation of mitochondrial respiration in cardiac and skeletal muscle cells in vivo obtained by using the permeabilized cell technique. Such an analysis in the framework of Molecular Systems Bioenergetics shows that the mechanisms of regulation of energy fluxes depend on the structural organization of the cells and interaction of mitochondria with cytoskeletal elements. Two types of cells of cardiac phenotype with very different structures were analyzed: adult cardiomyocytes and continuously dividing cancerous HL-1 cells. In cardiomyocytes mitochondria are arranged very regularly, and show rapid configuration changes of inner membrane but no fusion or fission, diffusion of ADP and ATP is restricted mostly at the level of mitochondrial outer membrane due to an interaction of heterodimeric tubulin with voltage dependent anion channel. VDAC VDAC with associated tubulin forms a supercomplex, Mitochondrial Interactosome, with mitochondrial creatine kinase. MtCK, which is structurally and functionally coupled to ATP synthase. Due to selectively limited permeability of VDAC for adenine nucleotides, mitochondrial respiration rate depends almost linearly upon the changes of cytoplasmic ADP concentration in their physiological range. Functional coupling of MtCK with ATP synthase amplifies this signal by recycling adenine nucleotides in mitochondria coupled to effective phosphocreatine synthesis. In cancerous HL-1 cells this complex is significantly modified: tubulin is replaced by hexokinase and MtCK is lacking resulting in direct utilization of mitochondrial ATP for glycolytic lactate production and in this way contributing in the mechanism of the Warburg effect. Systematic analysis of changes in the integrated system of energy metabolism is also helpful for better understanding of pathogenesis of many other diseases.

© 2010 Elsevier B.V. All rights reserved.

1. General introduction

Quantitative analysis of complex systems of integrated energy metabolism needs the collection of vast amount of reliable experimental data and use of mathematical models for analysis and prediction of system behavior. Important data can be obtained by studies of intact cells and organs such as the heart, skeletal muscles or brain by using such methods as imaging, biochemical analysis, nuclear magnetic resonance, including saturation transfer and especially by isotopic tracer method [1–3]. However, these methods usually give general information, not sufficient for revealing details of interactions between cellular components and for quantitative analysis of functional consequences of these interactions. This information can be easily obtained by permeabilized cell technique which application in combination with image analysis, kinetic methods and modeling is very useful and informative [4,5]. The aim of this review article is to describe and analyze experimental data obtained in our laboratories by this method in studies of the regulation of metabolic fluxes and respiration in muscle and brain cells, with main focus on the regulation of mitochondrial respiration in cardiac cells under normal physiological conditions when the heart function is governed by the Frank–Starling law [6,7]. We take advantage of the availability of the cells of cardiac phenotype with very different cellular organizations, such as adult isolated cardiomyocytes and cultured continuously dividing cancerous HL-1 cells [8]. Comparative studies of these cells gave us important information on the structure–function relationship in determining the mechanisms of regulation of respiration and integrated intracellular energy metabolism in cells [9,10]. Finally, we show that systemic analysis of the integrated
cellular energy metabolism in the cells helps to understand pathogenetic mechanisms of several diseases, such as cancer and heart insufficiency.

2. Mitochondrial arrangement in adult cardiomyocytes versus HL-1 cells

By its nature, the contraction process needs very precise structural organization of sarcomeres of muscle cells [11]. Mitochondria in adult cardiomyocytes are located regularly at the level of A-band of sarcomeres, their positions are determined by their interactions with the cytoskeleton and the sarcoplastic reticulum [12-17]. Recent studies have also shown that the precise, cell type-dependent organization of mitochondria, and numerous interactions between these organelles and other cellular structures, play a fundamental role in regulations of mitochondrial function [9,13,14,18-21]. In cardiac and skeletal muscles regularly arranged intermyofilibrillar mitochondria [15] interact with other intracellular systems like the cytoskeleton and sarcoplastic reticulum [12-14,17,22]. This type of organization provides a bioenergetic basis for contraction, recruiting cytoskeletal proteins, controlling both mitochondrial shape and arrangement in the cell within Intracellular Energetic Units [13,14]. Importantly, the mitochondrial interactions with various cytoskeletal proteins (desmin, vimentin, tubulin or plectin) have been suggested to be directly involved in the modulation of mitochondrial function [17-20,23-25]. Firm evidence for such a role has been found for tubulin (see below). These interactions control evidently also mitochondrial dynamics and movement of mitochondria in the living cells [26]. In our recent quantitative studies of these connections in adult rat cardiomyocytes and in cultured continuously dividing non-beating (NB) HL-1 cells with differentiated cardiac phenotype, mitochondria were stained with MitoTracker® Green and studied by fluorescent confocal microscopy [26]. High speed scanning (1 image every 400 ms) revealed very rapid fluctuation of positions of fluorescence centers of mitochondria but no mitochondrial fusion or fission in adult cardiomyocytes (Fig. 1F). These fluctuations followed the pattern of random walk movement within the limits of the internal space of mitochondria, probably due to transitions between condensed and orthodox configurational states of matrix and inner membrane [26]. In contrast, HL-1 cells with differentiated cardiac phenotype do not exhibit the strictly regular mitochondrial distribution typical for rat cardiac cells (Fig. 1A-E). In these cells, mitochondria can be heterogeneous, highly dynamic and motile, undergoing continual fission, fusion and fast intracellular displacements at a velocity of 0.1-0.2 μm/s [8,26]. Thus, mitochondrial fusion or fission was seen only in cancerous NB HL-1 cells (Fig. 1) but not in adult cardiomyocytes. The differences observed in mitochondrial dynamics are related to distinct specific structural organization and mitochondria-cytoskeleton interactions in these cells. It will be shown below that strikingly different intracellular organization and dynamics of mitochondria in adult cardiomyocytes and HL-1 cells are responsible for their remarkably different functional parameters.

3. Differences in the mechanisms of regulation of mitochondrial function in vitro and in vivo, factor X hypothesis

Rapid development of bioenergetics during the last 60 years was possible due to studies of mitochondria and their composition and components in an isolated state [27]. These studies resulted in establishing the Mitchell's chemiosmotic theory of oxidative phosphorylation and rotary mechanism of ATP synthesis, two fundamental bases of modern bioenergetics [27]. In the studies of metabolism in whole cells and organs, progress was made first by rapid freezing of tissue and subsequent biochemical analysis of extracts [28], and then by in vivo 31P NMR spectroscopy [2,29]. These two methods when applied for studies of cardiac metabolism resulted in the discovery of the metabolic homeostasis of the heart, expressed as a constancy in concentrations of ATP, PCr and creatine, despite large variations in work load, in the myocardium [28,29]. Now the principal but unsolved question is whether one can explain the mechanisms of regulation of integrated energy metabolism of the cells in vivo by behavior of mitochondria in isolated state, in vitro? Very often, the answer a priori has been yes [30-35], leaving unanswered the question how intracellular organization and multiple interactions between cellular structures may influence the mechanisms of regulation of energy fluxes. Usually these interactions are simply ignored. However, theories based on simple extrapolation of data from studies on mitochondria in vitro fail in attempts to explain the metabolic homeostasis of cardiac cells under conditions of Frank-Starling law [7,10]. One of these simple theories, still very popular and actively used, assumes that mechanisms of regulation of mitochondrial respiration in vivo and in vitro by ADP are very similar; that mitochondria in vivo behave as in an homogenous medium and that cytoplasmic ADP is in equilibrium with the CK reaction [30-35]. Concentration of cytoplasmic ADP in equilibrium with CK reaction in heart cells under condition of metabolic stability is about 50–100 μM [36]. Taking into account that for isolated mitochondria the Km value for ADP is only 8–10 μM, one can see that no regulation of respiration by cytoplasmic ADP at its concentrations of 50–100 μM is possible, since adenine nucleotide translocator, ANT, is saturated by ADP and maximal respiration rate should always be observed. This is not the case, however, as it is known from classical studies in heart physiology that cardiac oxygen consumption increases linearly with elevations in workload (ATP hydrolysis) [6,37]. Moreover, under conditions of stable ATP, PCr and creatine concentrations, characteristic of energy metabolism of cardiac cells, the ADP concentration calculated from CK equilibrium should also be stable and should not respond to changes of respiration rate. In a similar way free Pi concentration and related parameters such as free energy of ATP hydrolysis (calculated from total metabolite contents and CK equilibrium) should not change under condition of metabolic stability. Thus, the main counter-arguments to the CK equilibrium theory – the phenomenon of metabolic stability of cardiac muscle and metabolic aspect of Frank-Starling law of the heart [29,37-40] – are uncontested. Veech et al. established the CK equilibrium experimentally only in the resting (noncontracting) muscle [41]. In the working muscle, creative kinases within the PCr phosphotransfer network function mostly in a non-equilibrium state, especially at elevated workloads [7,42].

In order to explain the regulation of mitochondrial respiration under conditions of metabolic stability and at the same time assuming the CK equilibrium, the theory of parallel activation by Ca2+ was proposed and continued to be supported [43-47]. According to this theory, the increase of cytoplasmic Ca2+ during excitation-contraction coupling cycle activates ATP hydrolysis in myofibrils, and simultaneously three dehydrogenases of Krebs cycle in mitochondrial matrix increasing production of NADH and FADH2 by push mechanism. The oxidation of the latter increases electron flow through the respiratory chain, generates the proton motive force and drives ATP synthesis [27,48]. Ca2+ is thought also to activate directly F1Fo-ATPase and complex I [43-49-52]. However, the parallel activation theory still does not fit with the requirement for the main signal of coordination of energy metabolism in cardiomyocytes recently formulated by O’Rourke [48]. According to O’Rourke’s principles, the variations of cytoplasmic [Ca2+] have to correspond to changes in workload, ATP consumption and respiration. This condition is not fulfilled, because the intracellular Ca2+ transients do not change during the length-dependent activation of sarcomere (mechanism on which is based the Frank-Starling’s law) [53-55]. Thus, the Frank-Starling mechanism puts into question the viability of the theory of parallel activation of contraction and respiration by Ca2+. Regulation of respiration by Ca2+ seems to explain the adrenergic activation of oxidative phosphorylation [43,47,49,52-56].
Fig. 1. A Enhanced confocal images of the mitochondrial network in NB HL-1 cells. Mitochondria were stained with MitoTracker® Green (in white). In details B, C, D and E, modifications of mitochondria network as a function of time t are depicted. Indeed, mitochondria are very dynamic undergoing continual fission and fusion events usually forming long and rapidly moving filament-like structures. F Visualization of positions of mitochondrial fluorescent mass centers in a cardiomyocyte over a long time (total duration 100 s) of rapid scanning: movements of fluorescence centers are limited within internal space of mitochondria. Positions of the fluorescence centers were stacked as a function of time. These fluorescence centers (which are assimilated to the center of mitochondria in cardiomyocytes) are shown as small yellow spheres. The position of fluorescent centers was superimposed with a reference confocal image of MitoTracker® Green fluorescence (in grey) showing mitochondrial localization. Note that the fluorescence centers are observed always within the space inside the mitochondria, but from mitochondrion to mitochondrion the motion pattern may differ from very low amplitude motions (pink frame) to wider motions distributed over significant space but always within the internal space of a mitochondrion (blue frame). Reproduced from Beraud et al. with permission [20].

63), but not the feedback regulation of respiration by workload changes during cardiac contraction under physiological conditions of action of Frank-Starling law [7,64].

To find a solution to this important problem of metabolic studies, an application of the principles of Systems Biology is very helpful. One of the main principles of Systems Biology is that interactions between
system's components lead to new system level properties which are absent when the components are isolated and which explain the mechanisms of functioning of the system, its biological function [10,65–67]. The permeabilized cell technique, in combination with kinetic analysis, mathematical modeling and whole cell and organ studies mentioned above is one of the principal methods of Molecular System Bioenergetics [10,67].

4. In vivo kinetics of regulation of respiration, central role of MCK

Kümmel was first to apply the permeabilized cell technique for studies of cardiac energy metabolism [68] and to discover that the quantitative characteristics of regulation of mitochondrial respiration – apparent Km for exogenous ADP – are very different in vitro and in permeabilized cardiomyocytes in situ. The latter exceeds the former by order of magnitude, showing significantly decreased affinity of mitochondrial respiration for exogenous ADP in vivo. This result was then confirmed in very many laboratories (Table 1). Detailed studies of this phenomenon in our laboratories led to the conclusion that it is the result of an interaction of mitochondria with cytoskeletal components, resulting in restricted permeability of the voltage dependent anion channel VDAC in mitochondrial outer membrane for ADP and also related to the specific structural organization of the cell by the cytoskeleton resulting in regular arrangement of mitochondria with surrounding structures and MgATPases into Intracellular Energetic Units (ICEUs) in adult cardiomyocytes and oxidative muscle cells [13]. High apparent Km for exogenous ADP in permeabilized cells is tissue specific, observed for cardiac fibers and isolated cardiomyocytes even after extraction of myosin (ghost fibers), and in fibers from slow twitch oxidative (but not fast twitch) glycolytic skeletal muscle, its value is high also in permeabilized hepatocytes and synaptosomes (Table 1). This tissue specificity excludes any possibility of explaining high Km values for ADP by long diffusion distance or specific effects of saponin, a detergent used for permeabilization [45], sometimes proposed in literature and critically already analyzed before [69]. The value of this parameter decreases significantly after treatment of cells with a small amount of proteolytic enzymes in a low concentration, showing the role of some proteins sensitive to this treatment, in control of mitochondrial responses to ADP in vivo (Table 1). This hypothetical protein was given the name “Factor X” and assumed to be separated from mitochondria during isolation procedure [70], as shown in Fig. 2. The initial hypothesis was that this protein controls the permeability of outer mitochondrial membrane for ADP [70]. Thus, the high apparent Km for exogenous ADP in regulation of mitochondrial respiration is a system level property, depending on the interaction of mitochondria with other cellular structures. Comparison of isolated mitochondria, permeabilized cardiomyocytes and permeabilized HL-1 cells confirms this conclusion and shows directly the independence of the mechanism of regulation of respiration upon structural organization of the cell. Fig. 3 shows that isolated cardiomyocytes, where the mitochondria are fixed, with very regular “crystal-like” arrangement at the level of sarcomeres, and where no fusion into reticular structures is possible and only configuration of mitochondrial inner membrane changes rapidly (Fig. 1F), apparent Km for exogenous ADP is very high, in agreement with the data in Table 1. In contrast, in permeabilized HL-1 cells, where the mitochondria are very dynamic and undergo continuous fusion and fission (Fig. 1A-E), the apparent Km for exogenous ADP is very low and close to that in isolated mitochondria [9].

Further important information was obtained when mitochondrial creatine kinase, MCK was activated by creatine. These experiments confirm and clearly demonstrate that the role of mitochondrial outer membrane in adenine nucleotides compartmentation and the functional coupling between MCK and ANT is much more important in vivo where the mitochondria interact with intracellular surrounding environment forming the unitary structure–functional organization of energy metabolism – Intracellular Energy Units (ICEUs) [13]. During permeabilization the cytoplasmic soluble enzymes not bound to the structures such as many glycolytic enzymes and MM-CK are released into the solution, but MCK stays in the mitochondrial intermembrane space firmly fixed by lysis–cardiopin interactions at the outer surface of inner membrane in the vicinity of ANT [71,72]. Table 1 shows that the addition of creatine significantly decreases the apparent Km for exogenous ADP by increasing the rate of recycling of ADP in mitochondria in MCK and oxidative phosphorylation reactions functionally coupled via adenine nucleotide translocator, ANT.

One of the most fruitful approaches in these studies was a demonstration of the role of local ADP concentrations in activating oxidative phosphorylation by applying the powerful ADP trapping system consisting of high activities of pyruvate kinase, PK, and phosphoenolpyruvate, PEP, capable of capturing and phosphorylating all ADP in soluble phase of the cytoplasm or medium equilibrated with it. Fig. 4 demonstrates how this system was used in recent experiments [73]. In the presence of PK–PEP system ADP produced in MgATPase and myofibrillar MM-CK reactions is trapped and rephosphorylated into ATP, and respiration is activated only due to local ADP produced in the MCK reaction (Fig. 4A). Fig. 4B shows that under these conditions MtCK maximally activates respiration and PK–PEP are not able to trap the ADP recycling in mitochondrial coupled reactions behind the outer mitochondrial membrane (MOM). It was shown in parallel experiments that in the in vitro system containing isolated mitochondria the PK–PEP system can trap significant amount of ADP produced by MCK [73]. These remarkable

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$K_{m}^{ADP}$ (μM)</th>
<th>$K_{m}^{ADP}$ (μM)</th>
<th>$K_{m}^{ADP}$ (μM) after treatment with tropinin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart tissue homogenate</td>
<td>22±16</td>
<td>36±16</td>
<td></td>
<td>[191]</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>329±50</td>
<td>356.5±5.6</td>
<td></td>
<td>[192]</td>
</tr>
<tr>
<td><em>Glen</em> cardiomyocytes</td>
<td>200±250</td>
<td>200±250</td>
<td></td>
<td>[23]</td>
</tr>
<tr>
<td>Skinned cardiac fibers</td>
<td>200±25</td>
<td>200±25</td>
<td></td>
<td>[23]</td>
</tr>
<tr>
<td><em>Glen</em> cardiac fibers</td>
<td>200±25</td>
<td>200±25</td>
<td></td>
<td>[23]</td>
</tr>
<tr>
<td>Permeabilized Skinned cardiac fibers</td>
<td>200±25</td>
<td>200±25</td>
<td></td>
<td>[23]</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>110±11</td>
<td>25±1</td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td>Skinned fast twitch skeletal muscle fibers</td>
<td>7.5±0.5</td>
<td>8–12</td>
<td></td>
<td>[200]</td>
</tr>
<tr>
<td>Rat heart isolated mitochondria</td>
<td>17±10</td>
<td>136±4.4</td>
<td></td>
<td>[194]</td>
</tr>
<tr>
<td>Rat brain isolated mitochondria</td>
<td>13±2.6</td>
<td>17.6±1</td>
<td></td>
<td>[191]</td>
</tr>
</tbody>
</table>

* The values of this parameter, apparent Km for exogenous ADP is always much higher in permeabilized isolated cardiomyocytes and in skinned cardiac fibers. The equality of Km (ADP) in these two types of permeabilized preparations is always a necessary criterion for evaluation of quality of preparation when skinned fibers are used [45,59,191,193,194]. In opposite cases, as sometimes published in literature [204], for critical review see [69], the preparations are damaged, but there is no reason to blame the technique of skinned fibers so well used in many laboratories, for failures of authors to use it correctly [204].
Fig. 2. Original hypothesis of the connection of mitochondrial outer membrane (MOM) and cytoskeleton in vivo, where the outer membrane VDAC channel ("porin") was assumed to be controlled by some cytoplasmic factor "X", which is lost during mitochondrial isolation, and therefore the (MOM) becomes in vitro absolutely permeable for ADP. Reproduced from Saks et al. with permission [70].

differences between mitochondria in vivo and in vitro were seen again in kinetic experiments described in Fig. 4C-E. After permeabilization, MgATP was first added to activate the intracellular MgATPases producing endogenous ADP that stimulates respiration (Fig. 4C). The PK-PEP system was then added to trap this endogenous ADP with an expected decrease in respiration rate. Finally, creatine was added stepwise to study the role and kinetics of the MtCK reaction in regulation of respiration in vivo in a situation modeling some characteristics of intracellular milieu. Most remarkably, creatine addition to permeabilized cardiomyocytes rapidly activates respiration up to a maximal value (Fig. 4C). Results of the kinetic analysis of this action are described below. However, when isolated heart mitochondria were used, creatine addition increased respiration rate to only about half of its maximal value (Fig. 4D) meaning half of ADP produced by MtCK leaked out through the mitochondrial outer membrane and was trapped by the PEP-PK system. Table 2 shows that the respiratory parameters — maximal respiration rates in the presence of ADP or MgATP + creatine are similar in isolated heart mitochondria and permeabilized cardiomyocytes when calculated per cytochrome aa3 content. Thus, the effect of PEP-PK on the respiration of isolated mitochondria is due to the leak of ADP via mitochondrial outer membrane. Experimental studies of kinetic properties of MtCK in isolated cardiac mitochondria and mathematical modeling of these properties [74–77] showed that they are dependent on functional coupling of MtCK with oxidative phosphorylation via ANT. The apparent constant of dissociation of ATP from its tertiary complex with MtCK decreases 10 times in the presence of activated oxidative phosphorylation [78]. This strong affinity of MtCK for ATP disappears when MtCK is detached from mitochondrial membranes [79]. Mathematical modeling of these effects showed that it is explained by direct channeling of ATP from ANT to MtCK [74,80]. However, ADP produced by MtCK can either be taken back into the matrix by ANT or leave intermembrane space if the outer membrane is easily permeable (see Fig. 4A). This is observed in isolated mitochondria (Fig. 4D) but not in permeabilized cardiomyocytes (Fig. 4B and C). Fig. 4E shows that in permeabilized fibers from human skeletal muscle m. vastus lateralis MtCK also effectively regulates respiration. In similar experiments with permeabilized fibers from biopsy samples of human m. vastus lateralis the apparent Km for exogenous ADP was found to be high but decreased significantly in the presence of creatine [81,82], supporting the conclusion that in oxidative skeletal muscles the ADP diffusion into mitochondrial intermembrane space is restricted. These reliable experimental results contradict the theoretical conclusion of mitochondrial ADP ultrasensitivity in these muscles made by Jeneson et al. [32] from analysis of PCr recovery after exercise on the basis of the assumption of CK equilibrium: experimental value of apparent Km for ADP was close to 200 μM [81] while theoretical calculations gave 22 μM [32]. Once again, the assumption of the CK equilibrium is not sufficient to explain the experimental data. To assess the role of structural organization in determining the mechanisms of regulation of oxidative phosphorylation, we have taken advantage of the comparison of cardiomyocytes from an adult heart with HL-1 cell culture developed from mouse atrial cardiomyocytes and expressing cardiac phenotype [8,83]. The HL-1 cells are characterized by entirely different kinetics of regulation of respiration.

![Graph](image-url)

Fig. 3. Different kinetics of regulation of the respiration in permeabilized adult cardiomyocytes and non-heating (NB) cardiac tumor HL-1 cells. For normalization, the respiration rates were expressed as fractions of the maximal rates, Vmax, found by analysis of experimental data in double-reciprocal plots. Reproduced from Ammann et al. with permission [9].
by ADP (see Fig. 3). Moreover, in the permeabilized HL-1 cells the
creatine effect on respiration was not observed (Fig. 4F). It was
recently shown that MCK is not expressed in these cells, but only
the BB isoform of CK can be seen, in contrast to rat heart cells, where BB
isoform is in trace amounts and the major forms are MCK and MMCK
isoforms, and also some amount of hybrid form MB is seen [84].

Remarkably, in both cells two hexokinase isoforms are present [84].
Measurements of enzyme activities in both these cells showed that CK
activity is manifold decreased in HL-1 cells as compared to
cardiomycocytes, while the activity of hexokinase is significantly
increased in HL-1 cells (Fig. 5). Significance of these findings for
cancer cell bioenergetics is discussed below.

Fig. 4. (A) The scheme represents a mitochondrion in situ, in a permeabilised cardiac cell. The mitochondrial outer membrane (MOM) is less permeable than in isolated
mitochondrion, due to the interactions of VDAC with cytoskeleton protein tubulin. Exogenous ATP is hydrolyzed by cellular ATPases into endogenous extramitochondrial ADP and
inorganic phosphate (Pi). Mitochondrial (MCK) and non-mitochondrial MM creatine kinases (cytosolic, myofibrillar, SERCA, and sarcolemmal) activated by creatine in the presence
of ATP, produce endogenous intra- and extramitochondrial ADP. The system is supplemented with phosphoenolpyruvate (PEP) and pyruvate kinase (PK) which remove
extramitochondrial ADP and continuously regenerates extramitochondrial ATP. Intramitochondrial ADP produced by MCK forms microcompartments within the mitochondrial
space (IMS) and is re-imported into the matrix via adenine nucleotide translocase (ANT) due to its functional coupling with MCK. Reproduced from Guana et al. [73] with
permission. (B) Respiration rates of permeabilized cardiomyocytes. Respiration was activated by addition of MgATP (5 mM) and creatine (20 mM) resulting in activation of the MCK
reaction with local production of intramitochondrial ADP. Addition of 20 IU/ml PK in the presence of PEP (5 mM added into medium before) did not change significantly the
respiration rate because of the inaccessibility of compartmentalized, in mitochondrial intermembrane space, ADP for PK-PEP system. (C) The experimental procedure used for
complete kinetic analysis of MCK in mitochondria in situ (permeabilized cardiomyocyte). First, addition of MgATP induces production of endogenous ADP in MgATPase reaction.
Secondly added PEP-PK trap all extramitochondrial free ADP inducing decrease of respiration rate, but not to initial level, due to structural organization of IEC. Under this
conditions addition of creatine in different amounts rapidly activates the MCK reaction. The oxidative phosphorylation is stimulated mostly by intramitochondrial ADP, produced by
MCK Reaction, which is not accessible for PEP-PK. Adapted from [73]. (D) Respiration rates of isolated mitochondria stimulated by increasing amounts of creatine in the presence of
ATP (i.e. activated MCK reaction) and the absence of extramitochondrial ADP (consumed by the PEP-PK reaction). Adapted from [73]. (E) Respiration of permeabilized fibers from
human skeletal muscle vastus lateralis is prepared from biopsy samples of healthy volunteers in the presence of 2 mM malate and 5 mM glutamate as substrates. Addition of 2 mM MgATP
activates respiration due to production of endogenous MgADP in ATPase reaction. Pyruvate kinase (PK) in the presence of 5 mM phosphoenolpyruvate (PEP) decreases respiration
rate due to removal of extramitochondrial MgADP. Creatine in the presence of MgATP activates mitochondrial creatine kinase (MCK) reaction of production of endogenous
intramitochondrial MgADP which rapidly activates respiration up to the maximal rate, that showing that mitochondrial ADP is not accessible for PK-PEP system due to the limited
permeability of mitochondrial outer membrane in the cells in situ. Reproduced from Cherek thesis [201] with permission. (F) The absence of the stimulatory effect of creatine on the
respiration rate of non-heating (NB) cardiac tumoral HL-1 cells in the presence of PEP-PK system under conditions described above. Recorded respiratory rate is due to the stimulatory
effect. Adapted from [9].
5. Mitochondrial–cytoskeletal interactions, heterodimeric tubulin as factor X

Many experimental evidences discussed above point to the role of some cytoskeleton protein, factor "X" in regulating MOM permeability for adenine nucleotides. One of the real candidates for this role is tubulin. Carre et al., by using the immunoprecipitation method showed the association of tubulin with VDAC [85]. Appaix et al. demonstrated that selective proteolytic treatment of permeabilized cardiomyocytes by trypsin in low concentration, which decreases apparent Km for exogenous ADP, also results in the almost complete disappearance of immunolabeling of tubulin [19]. These results are reproduced in Fig. 6. Very recently, functional interaction of tubulin with VDAC was revealed by applying biophysical and oxygraphic methods by Rostovtseva et al. [86,87] and Monge et al. [88]. In experiments with VDAC reconstituted into planar phospholipid membranes the reversible voltage dependent partial blockage of channel by dimeric tubulin (in nanomolar concentration insufficient for polymerization and in the absence of GTP and Mg$^{2+}$) was observed. Under similar experimental conditions but without tubulin VDAC remains open up to 1 h [87]. Rostovtseva et al. proposed the model for tubulin–VDAC interaction in which the negatively charged C-terminal tail of tubulin penetrates into the channel lumen due to the interaction with a positively charged domain of VDAC close it [87].

The role of interaction of tubulin with VDAC in regulation of mitochondrial oxidative phosphorylation was studied directly by recording the respiration rates of isolated brain and heart mitochondria stimulated by exogenous ADP in the absence and presence of heterodimeric tubulin (Fig. 7). As was expected, the apparent Km for free ADP in isolated mitochondria was about 10–20 µM. In the presence of 1 µM tubulin the sensitivity of mitochondria for free ADP decreased: apparent Km increased to ~170 µM for the brain and ~330 µM for the heart mitochondria, respectively [87,88]. Creatine addition effectively decreased the apparent Km for ADP again (Fig. 8).

These studies allowed the factor X to be finally identified as the heterodimeric tubulin [86,87].

The effect of creatine and kinetic analysis of system described in Fig. 4C showed that VDAC cannot be completely closed in vivo. We analyzed the kinetics of respiration regulation by creatine and ATP in permeabilized cardiomyocytes in situ when respiration was stimulated by intramitochondrial ADP produced in MtCK reaction and extramitochondrial ADP was continuously consumed by the PEP–PK system (see Fig. 4A–C). These experiments modeled an interaction of mitochondria with glycolytic system in vivo. The apparent kinetics of
the MtCK dependent respiration regulation was found to be totally different from that seen in mitochondria in vitro [73]. In fact, there are three remarkable differences. First is the decrease in apparent affinity of MtCK for exogenous MgATP (apparent $K_m$ increased more than 100 times, Table 3) in mitochondria in situ as compared to in vitro. Second, the apparent constant of dissociation of creatine from the binary complex with MtCK (Kib) decreases about 10 times in mitochondria in situ, in permeabilized cardiomycocytes, as compared with isolated mitochondia. Third, the apparent affinity of MtCK for PCR is similar in vitro and in situ in permeabilized cells (Kip is about 1 mM) (Table 3). The decreased apparent affinity of MtCK in situ for extramitochondrial MgATP can be most probably due to the enhanced restriction of diffusion at the level of MOM (i.e. limited VDAC permeability) which induces the increase of adenine nucleotide micro-compartmentation within mitochondrial intermembrane space influencing the respiratory control of oxidative phosphorylation. The remarkably high affinity of MtCK in mitochondria in situ and PCR points to the absence of restriction of diffusion of these guanido substrates across MOM into intermembrane space where MtCK is located. Direct measurements of energy fluxes from the mitochondria into the surrounding medium (measured using high performance liquid chromatography (HPLC) for the same experiments) showed stable MgATP concentration though the experiment and progressive increase of PCR concentration was dependent on a stepwise increase in creatine concentration. The PCR/O$_2$ ratio was equal to 5.7 and close to the theoretical maximal P/O ratio under conditions similar to those in vivo [89]. These results show that PCR is the main energy flux carried out from mitochondria in permeabilized cardiomyocytes.

The selective regulation of barrier functions of MOM by cytoskeleton and functional coupling of MtCK with ANT is highly important for the structural and functional organization of energy metabolism and regulation of effective exchange of phosphoryl groups between two systems of recycling metabolites. ATP/ADP recycling is restricted mostly to mitochondrial intermembrane and matrix spaces, while GR and PCR are recycled between mitochondrial intermembrane and cytoplasmic spaces, resulting in energy transport from mitochondria into cytoplasm by freely diffusible PCR molecules via the system of compartmentalized CK reactions (Fig. 8). This figure shows the scheme of PCR/CK shuttle circuit in the brain, heart and skeletal muscle cells, in details described elsewhere [71,121,42,70–73,90]. The coupling between two metabolic cycles is realized in mitochondria by a supercomplex which we called Mitochondrial Interactosome, MI (Fig. 9). This complex is formed by ATP synthasome (a term proposed by Pedersen [91–94] and constituted by ATP synthase, ANT and Pi carrier) functionally coupled to MtCK [10,42,75,76,86,89,95–97], and VDAC with tubulin and some other regulatory proteins (Fig. 9). [85,95]. This unit can also include the super complex formed by the respiratory chain [98,99]. The role of Mitochondrial Interactosome is to ensure continuous recycling of adenine nucleotides in mitochondria, their transphosphorylation and metabolic channeling of ATP via ANT to MtCK, and back ATP, resulting in the export of free energy from mitochondria into cytoplasm as flux of PG. The functioning of this complex structure is best explained by the theory of vectorial metabolism and the vectorial ligand conduction, proposed by P. Mitchell [100]. Initially, this theory was developed to explain the organization of enzymes in supercomplexes allowing the scalar transport of electrons and the vectorial conduction of protons through the mitochondrial inner membrane to create the electrochemical potential [100,101]. Later, this concept was applied to the functioning of the phosphotransfer shuttle PCR/CK, AK [1,102,102], and to the transmission of [ADP] feedback signal from myofibril to mitochondria [10,70,89,90,104].

Mitochondrial Interactosome is an integral part of the creatine kinase phosphotransfer network (shuttle) [1042,90,105–108]. This network explains metabolic aspects of Frank–Starling law of the heart [7,108] and is quantitatively described by the mathematical model of compartmentalized energy transfer which describes non-equilibrium kinetics of creatine kinases functioning in opposite directions in mitochondria and myofibrils and takes into account the limited permeability of mitochondrial outer membrane for adenine nucleotides [104]. The model shows that cytosolic free ADP concentration may reach the levels of even 400 μM [73,61,104]. This value is markedly exceeds the free ADP levels calculated from the CK equilibrium constant (50–100 μM) [26]. This mathematical model also helps to explain the importance of the limitation of the permeability of the outer mitochondrial membrane in cardiac cells within the Mitochondrial Interactosome for the effective control of oxidative phosphorylation by one of intracellular factors — the cytoplasmic ADP. As we have seen above, the apparent $K_m$ for free ADP in mitochondria in situ (in permeabilized cardiac cells and fibers) is about 20 times higher than in isolated mitochondia. When MOM is permeable, as in isolated mitochondia, the respiration regulation by cytoplasmic ADP is impossible because of saturating ADP concentrations (50–400 μM) in Fig. 10. Under these conditions cytoplasmic ADP has no role in respiration regulation. According to O’Rourke’s principle mentioned above [48], changes in metabolic regulator should correlate with changes in workload and respiration rates. When ADP diffusion is restricted at the level of MOM, as it is in mitochondria in situ in permeabilized cardiomycocytes, the respiration rates become almost linearly dependent on cytoplasmic ADP concentrations when the latter changes in the range of physiologic concentrations up to 200–400 μM (shown by shaded area in Fig. 10). When MtCK is activated as it is in MI in vivo, the linear relationship between respiration rates and increase in free [ADP] is amplified and displaced towards the left region of Hemici

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mitochondria in vitro</th>
<th>Mitochondria in situ (permeabilized cardiomycocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_o$ nM O$_2$ min$^{-1}$ mg prot$^{-1}$</td>
<td>26.37 ± 7.93</td>
<td>7.53 ± 1.61</td>
</tr>
<tr>
<td>$V_o$ 2 mM oxalo, nM O$_2$ min$^{-1}$ mg prot$^{-1}$</td>
<td>187.94 ± 40.68</td>
<td>84.45 ± 13.85</td>
</tr>
<tr>
<td>[Cl-$\text{\text{a}}$] nM mg prot$^{-1}$</td>
<td>1.00 ± 0.012</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>$V_o$ MtCK, nM O$_2$ min$^{-1}$ nMol cy a$_3$</td>
<td>188 ± 39.93</td>
<td>178.23 ± 33.96</td>
</tr>
<tr>
<td>$V_o$ MtCK, nM O$_2$ min$^{-1}$ nMol cy a$_3$</td>
<td>197.90 ± 31.96</td>
<td>162.63 ± 26.87</td>
</tr>
</tbody>
</table>

Fig. 5. Enzyme activity profile in rat adult cardiomyocytes (CM), in non-beating HL-1 cells, Hexokinase (HK), fructose-6-phosphokinase (F6PK), lactate dehydrogenase (LDH), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and creatine kinase (CK) activities were measured and represented on a logarithmic scale. The means are presented ± SD. Adapted from [202].
Michaelis–Menten representation due to recycling of ADP in coupled reactions in MI (see Fig. 10). In other words, for effective regulation of respiration in dependence of workload in cardiac cells in vivo, changes in concentrations of cytosolic ADP (as calculated by the model) are necessary and sufficient only when MtCK is actively functioning within the coupled systems in Mitochondrial Interactosome. An interesting and important task will be to apply the methods of Metabolic Control Analysis in experiments with permeabilized cells with fully activated MtCK in the presence of the FEP-PK system (see Fig. 4) to measure the flux control coefficients of different components of Mitochondrial Interactosome to quantitatively characterize their role in control of respiration and energy fluxes. Calculation of flux control coefficients from the mathematical model of compartmentalized energy transfer [109] showed that other important signals in metabolic feedback regulation of respiration may be cyclic changes in Pi and PG/Cr ratio. Possible role of Pi in regulation of respiration was confirmed experimentally [110,111]. Both mathematical modeling and direct experimental determination of energy fluxes from mitochondria in permeabilized cardiomyocytes showed that under these conditions energy is carried into the cytoplasm mostly by phosphocreatine.
molecules [73, 89, 112]. Thus, there is clear separation of mass and energy transfer (by PCR and Cr) and information transfer (feedback metabolic signaling) [89].

The very remarkable observation is that physical activity changes the regulation of mitochondrial respiration via Mitochondrial Intera-
tactosome by increasing the value of apparent Km for exogenous ADP by a factor of 3 [81, 82, 113], while at the same time increasing the activity of MCK [81, 114]. As a result, the effect of creatine on respiration rate will be more significant [81, 82, 113]. The elucidation of the nature of changes induced in MI by physical exercise needs further experimental studies.

Mitochondrial Intertacontosome, as is shown in Fig. 9, helps us to explain also another classical observation in the history of bioenergetics. Belitzer and Tsybakova showed in 1939 [115] that creatine added to a well washed homogenate of pigeon pectoral muscle strongly increased oxygen uptake and production of phosphagen (as phosphocreatine, PCR, was called at that time) without any added adenine nucleotides present only in trace amounts. The efficiency coefficient of aerobic synthesis of phosphagen, the PCR/O2 ratio was between 52 and 7 [115]. This was one of the first determinations of

Fig. 7. Comparison of apparent Km for exogenous ADP in isolated heart mitochondria in three different conditions: control (mitochondria without tubulin and creatine), with tubulin 1 μM (two populations of mitochondria appeared) and with tubulin 1 μM and creatine 20 nM. The incubation with 1 μM tubulin was performed for 30 min at room temperature. The means are presented ± SD. Adapted from [203].

Fig. 8. Organization of compartmentalized energy transfer and metabolism in cardiac, skeletal muscle and brain cells and major routes of Cr metabolism in the mammalian body. The scheme shows the structural organization of the energy transfer networks of coupled creatine kinase (CK) reactions in mitochondria (1), at sarcolemmal membrane (2) and in myofibrils (3). The mitochondria, ATP-sensitive systems in sarcolemma and MgATPase of myofibrils are interconnected by creatine (Cr) and phosphocreatine (PCr) and energy transfer by the creatine kinase-phosphocreatine system. In brain cell systems energy transfer reactions are presented only by coupled reactions (1) and (2). Adenine nucleotides within local compartments 1, 2 and 3 do not equilibrate rapidly with adenine nucleotides in the bulk water phase. Right panels show confocal images of rat brain synaptosomes, cardiomyocytes and skeletal muscle. Mitochondria were labelled by Mitotracker Red or Mitotracker Green (50 nM). Very regular arrangement of mitochondria in striated muscles and fixed granular mitochondria in synaptosomes is seen. Creatine is not synthesized in these cells, but transported into cells by creatine transporter, CRT.
Table 3
Kinetic properties of MCK in situ in cardiac myocytes. The MCK reaction mechanism, built quasi equilibrium random type is characterized by two dissociation constants for each substrate as shown in the following scheme [73,88]:

Values of constants for isolated mitochondria are taken from the literature [78,137]. In isolated mitochondria the oxidative phosphorylation decreases dissociation constants of MgATP from MCK-substrate complexes suggesting the privileged uptake of all ATP by MCK. In mitochondria in situ in permeabilized cardiomyocytes the increase of apparent constants of dissociation of MgATP compared with in vitro mitochondria shows the decrease of apparent affinity of MCK in situ for extramitochondrial MgATP. The decrease of apparent constants of dissociation of creatine from MCK-substrate complexes suggests the increase of the apparent affinity of MCK for creatine in situ. The apparent constant of dissociation for PCr did not change in situ compared with isolated mitochondria. Reproduced from Gauze et al. with permission [73].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Isolated mitochondria</th>
<th>Mitochondria in situ (PEP-PK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X0 (MgATP), mM</td>
<td>X0 (MgATP), mM</td>
<td>X1 (Cr), mM</td>
</tr>
<tr>
<td>K1 (MgATP), mM</td>
<td>0.92 ± 0.09</td>
<td>1.5 ± 0.23</td>
</tr>
<tr>
<td>K2 (MgATP), mM</td>
<td>0.44 ± 0.08</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>K3 (MgATP), mM</td>
<td>1.94 ± 0.86</td>
<td>2.04 ± 0.14</td>
</tr>
</tbody>
</table>

6. Pathogenic mechanisms related to changes in organization of integrated energy metabolism

Dysfunction of integrated energy metabolism may be among leading mechanisms of pathogenesis of many diseases. A classical example of the importance of cellular organization of a complex system of energy metabolism is the Warburg effect: increase of lactate production in tumor cells in the presence of oxygen [116–119] first reported in the 1920s by Otto Warburg [118]. The conversion of glucose to lactate yields 2 mol of ATP per mole of glucose in comparison to 38 mol of ATP when glucose is completely degraded to CO2 and H2O. Thus, degradation of glucose to lactate yields only 5% of the energy available from glucose. This apparently senseless waste of energy prompted Warburg to postulate a defect in respiration in tumor cells as a cause for the increased "aerobic glycolysis" [116,118].

These and many other diseases are caused by cellular pathologies related to changes in mitochondrial structure and function, known as mitochondrial pathologies [119,120].

In normal cells competition between glycolysis and oxidation of fatty acids leads to their coordinated function with the aim to provide cells with less free energy into the adenylate system (∆GATP) from catabolic reactions [21] mostly to maintain cellular work, ion transport and biosynthesis. When workload increases, ATP production and respiration are increased due to feedback regulation via the CK system [7,21]. These pathways occur under aerobic conditions. Among the mechanisms limiting the glycolytic rate in normal cells are the intrinsic kinetic properties of soluble isoforms of hexokinase, HK, the inhibition of HK by the reaction product glucose-6-phosphate (G-6-P), and finally the inhibition by citrate via phosphofructokinase (PFK).

In cancer cells this mechanism of regulation is lost and substituted by other mechanisms of interaction between glycolysis and oxidative phosphorylation that result in the parallel activation of both systems [121]. Our studies on HL-1 cells, which are derived from mouse atrial cardiomyocytes but also carry some properties of cancer cells (such as unlimited proliferation) have been useful for understanding the metabolic changes associated with the development of tumor cell phenotype. As we have seen in Fig. 3, mitochondria in NB HL-1 cells exhibit very high apparent affinity for exogenous ADP similar to that of isolated mitochondria. In addition, creatine is unable to stimulate respiration of NB HL-1 cells (Fig. 4F) due to downregulation of mitochondrial MCK and cytosolic MM-MCK [84]. The only CK isomorph in cytosol of NB HL-1 cells was found to be BB-CK isomorph [84]. At the same time, metabolic profile of NB HL-1 cells is characterized by the prevalence of glycolytic enzyme activity, especially by that of HK and PK (Fig. 5). Total HK activity in NB HL-1 cells homogenate was increased by a factor of 5 in comparison with adult cardiomyocytes (Fig. 5) [122]. High activity of hexokinase is seen even after permeabilization of cells when only the activity of membrane-bounded enzymes can be measured [84]. Fig. 11 shows that glucose exerted a remarkable stimulatory effect on mitochondrial respiration in NB HL-1 cells, in competition with a negligible effect on respiration of permeabilized heart fibers [84]. Lack of stimulatory effect of glucose on oxygen uptake in the presence of ATP in permeabilized heart cells means that while hexokinase is expressed in cardiomyocytes [84], it is not bound to mitochondrial membrane where tubulin occupies binding sites near VDAC within Mitochondrial Interactosome. In cancerous HL-1 cells the Mitochondrial Interactosome structure is significantly modified (Fig. 12): tubulin has evidently given place to HK, and the absence of MCK allows all mitochondrial ATP to be captured for phosphorylation of glucose and stimulation of glycolytic lactate production. Thus, the Mitochondrial Interactosome typical for normal cardiomyocytes is replaced by another type of Mitochondrial Interactosome, in which mitochondrial bound HK substitutes for the role of MCK. These structural rearrangements result in a specific metabolic phenotype characterized by the replacement of creatine control of respiration, characteristic of adult cardiac cells, by that of glucose in NBHL-1, thus underlying the so-called "Warburg effect". As
it was described by Pederson et al., in cancer cells the over-expressed HK2 is bound to VDAC in the MOM [91–93,121,123–126] and via this coupling can exert control over oxidative phosphorylation. The apparent Km of membrane-bound HK2 for glucose is about 250 times lower than for soluble isoenzymes [127] and it is protected from the G6P product inhibition by its connection with VDAC. Glycolytic ATP synthesized during lactate production is used in biosynthetic pathways for growth and proliferation [125,126]. In this way, it seems that the “energetic” role of mitochondria in cancer cells is reduced to maintain glycolysis. However, oxidative phosphorylation, although suppressed due to decreased biogenesis of mitochondria is still preserved, in order to facilitate glycolysis. Currently, it is not clear how the transformation from normal to cancer metabolism proceeds, how normal mechanisms of coordination between glycolytic and oxidative networks degrade into the most primitive model of glycolytic energy metabolism. Groft et al., in 2009 tried to answer this question by studying the mechanisms of Warburg phenotype development during immortalization of mouse fibroblast cells (H-RasV12/E1A). This process was shown to be dependent on the number of culture-passages and characterized by the increase in glucose-to-lactic flux and cellular oxygen consumption associated with the decreased TCA and oxidative phosphorylation activity [128].

Most of the other pathologies are related to defects in the respiratory chain complexes and lead to decreased ATP production as a result of either inhibition of respiration or uncoupling of phosphorylation and respiration [120]. Among pathogenic mechanisms related to changes in mitochondrial functions is the opening of mitochondrial permeability transition pore, PTP, induced by calcium overload of cells and accelerated by production of reactive oxygen species, ROS [129,130]. PTP opening leads to cell death by necrosis, and this is the most common mechanism related to ischemic and reperfusion injuries [129,130]. ROS production by chemical (non-enzymatic) reaction of molecular oxygen with reduced electron carriers in the complexes I and III of the respiratory chain is considered also as the major mechanism of ageing and cancerogenesis [131]. ROS production consumes from 1 to 5 % of oxygen supplied to the cells and cannot be avoided, but can be controlled by means of controlling the red-ox state of the respiratory chain [131]. Both ROS production and PTP opening can be effectively controlled and inhibited by the MCK reaction which is functionally coupled to the adenine nucleotide translocase, controls respiration and ATP-ADP recycling in mitochondria and by this way controls the red-ox state of the respiratory chain and also the conformational state of mitochondrial carriers important for PTP opening [97,132].
7. Parameters of PCr/Cr system as diagnostic means

In the cells with high energy demand the creatine kinase system is responsible for facilitating energy supply to local pools of the ATP near ATPases and ATP-sensitive channel in sarcolemma (see Fig. 8), thus controlling both contraction and excitation-contraction coupling [17,104,22,88,105,108,132–143]. Changes in this central system of energy supply either due to alteration of creatine kinase isoenzyme composition, activity and mechanisms of interaction with ATP producing and consuming systems, or decrease of total creatine content may lead to serious pathology of the heart, skeletal muscle and nervous system [133–135,143]. Clinical studies of patients with severe cardiomyopathy by non-invasive nuclear magnetic resonance imaging and spectroscopy revealed a very high diagnostic value of parameters of functioning of creatine kinase system — the PCR/ATP ratio for prognosis of patient’s mortality rate and thus survival [133]. Alternatively, dietary supplementation of creatine has been shown to be an effective mean of pharmacological treatment and protection of patients with muscular and neurodegenerative diseases [105,135,136,139–141]. Systems biology approaches to studies of these integrated processes of energy metabolism in normal cell life and in their pathology may have an utmost importance for clinical medicine in the near future [7,144,145].

In myocardial infarction and in heart failure, a rapid decrease of PCr content occurs due to lack of oxygen supply and pathological changes in the creatine kinase system [108,133–137]. Total ATP content usually changes very slowly and its changes, as well as changes in the free energy of ATP hydrolysis calculated from total metabolites’ contents, are dissociated from the rapid fall of the cardiac contractile force [28,146], total depletion of ATP resulting in contracture of the heart muscle [147]. A rapid decline in heart contractile function in hypoxia and ischemia is most likely to be related to changes in compartmentalized energy transfer systems, leading to decreased regeneration of ATP in functionally important cellular compartments, as shown in the scheme in Fig. 8. Firstly, the rapid decline in ATP regeneration in subsarcolemmal area results in changes of ion currents across this membrane and thus in shortening of action potential [1,137], and secondly, the rapid decline in ATP regeneration in myofilibrillar microcompartment due to lack of phosphocreatine slows down the contraction cycle [108,137]. Similar but slower changes are observed in chronic cardiac and skeletal muscle diseases [148–157]. In concord with this conclusion are the results published by Weiss et al. [154] showing that cardiac ATP flux through CK is reduced by 50% in cases of human heart failure in the absence of reduction of ATP stores. Local phosphotransfer networks in the subsarcolemmal area are an important part of the membrane sensors of the cellular energy state also in brain cells [136,139–141], explaining the dependence of functional state of these cells on phosphocreatine supply, and thus the central importance of the PCr/Cr system. In brain cells ubiquitous mitochondrial creatine kinase is co-expressed with BB isoenzyme localized both in cytoplasm and at the cell membrane (coupled creatine kinases 1 and 2 in the Fig. 8). Alterations of these systems are observed in many neurodegenerative diseases [139,140].

The content of phosphocreatine, central energy carrier in the cells, depends both on its continuous regeneration in mitochondrial creatine kinase reaction coupled to oxidative phosphorylation, and on the total creatine content which in significant part depends on the import of creatine (not produced in muscle or brain cells) from blood by creatine transporters in the cell membrane. This explains the beneficial effects of dietary supplementation of creatine on the energy metabolism and functional state of skeletal muscle and brain cells.

![Fig. 10](image_url) Henri-Michaelis–Menten hyperbolic representation of kinetics of respiration regulation by free ADP in isolated heart mitochondria and permeabilized cardiomyocytes (in the absence or presence of creatine). The grey area delimits physiologic range of changes in cytosolic [ADP] taken from the model of compartmentalized energy transfer in cardiomyocytes [104]. In isolated mitochondria (curve Α), no regulation of respiration is possible because of the saturating free [ADP] for the minimal workload. When the ADP diffusion is restricted as in mitochondria in situ in permeabilized cardiomyocytes (curve B), the respiration rates become linearly dependent on ADP concentrations in their physiological range. In this interval of quasi-linear dependence under physiological conditions the activating effect of ADP can be amplified by creatine (curve C), due to activation of coupled MK. The resulting apparent Km for cytoplasmic ADP is significantly decreased and respiration rate increased.

![Fig. 11](image_url) Oxygenic analysis of coupling of hexokinase (HK) to OxPhos in permeabilized HL-1 cells and rat left ventricular fibers. The thin line corresponds to respiration rates of permeabilized rat left ventricular fibers or cardiomyocytes; the thick line shows respiration rates of HL-1 cells. Ten millimolars of glucose activates HL-1 cell respiration in the presence of 0.1 mM MgATP due to the endogenous ADP production in HK reaction. Similar amount of glucose cannot stimulate respiration rate of permeabilized fibers or cardiomyocytes. ADP — 2 mM MgADP, Atr — 0.1 mM, atracyloside. Reproduced from Emre et al. with permission [84].
especially in patients with neurodegenerative diseases [139-141,158-160].

Dissociation of total content of ATP from cell function was recently clearly demonstrated by O'Connor et al. [142], who showed that it is the Pcr/Cr system which sustains localized ATP-dependent reactions during actin polymerization in myoplasm fusion. Myoplasm treated with exogenous creatine showed enhanced intracellular Pcr stores without any effect on ATP levels. This increase in Pcr induced the myoplasm fusion and myotube formation during the initial 24 h of myogenesis. During this time BB-Cr became localized and after 36 and 48 h was found close to the ends of the myotubes [1-42]. Actin polymerization is critical for myoplasm fusion and occurs with involvement of ATP both during the addition of actin monomers to the growing ends of filaments and the dissociation of monomers at the tail. It is this localized ATP which is rapidly regenerated by BB-Cr at the expense of Pcr, and it seems that the formation of these ATP microdomains is a dynamic process during actin cytoskeleton remodeling. Local injection of creatine into injured skeletal muscle increased the growth of regenerating myofibers from satellite cells via differentiation and fusion of myofiblasts [142]. All these results add new insight into the functioning of the Pcr/CK system in muscle cells, showing its new role in energy supply for cytoskeleton remodeling. These results may help to better explain the therapeutic effects of creatine supplementation [105,135,136,143,158-160].

Intensive and numerous studies have been carried out on transgenic mice with knockout of different CK isoenzymes, or enzymes responsible for creatine metabolism and transport (reviewed in [149,152,161,162]). In spite of multiple adaptive mechanisms — activation of alternative phosphotransfer pathways, such as adenylate kinase shuttle [161,163], structural changes in the cells and increase of oxidative capacity of skeletal muscle [152,161], and many others [164], significant functional and metabolic changes especially related to calcium metabolism and contractile performance have been observed in these experiments [162,164-167]. Thus, Momken et al. have reported that double knockout of MtkCK and MM-Cr very significantly impairs the voluntary running capacity of mice [165]. Knockout of enzymes of creatine biosynthesis in mice resulted in significantly reduced responses to inotropic stimulation [162]. Similarly, the hearts of rats treated with guanidinopropionic acid performed much less pressure-volume work [168]. Most interestingly, recent works from Neubauer's laboratory have shown that overexpression of creatine transporter and supranormal myocardial creatine contents lead to heart failure [169,170]. In these hearts creatine content is increased by more than a factor of 2 [169].

Most interestingly, these experiments put into evidence the importance of the PCr shuttle: heart failure may be due to the formation of dead-end complex CMGADP.Cr formation [171] and inhibition of Pcr utilization for local ATP regeneration.

In summary, data obtained in experiments with CK knockout mice are in concordance with our conclusion made in this work and before [7,10,42,107,136,137] that muscle (and other) cells are viable without MtkCK and other CK isoenzymes, as HL-1 cells, but Pcr-Cr and other phosphotransfer pathways are necessary for effective energy transfer and metabolic regulation at higher energy demand, and thus for survival under stress conditions. An important observation is that exercise training results in cytoskeleton remodeling, including changes in Mitochondrial Interactosome and increased efficiency of energy transfer via Pcr-Cr pathway [81,82,113,114]. By analogy, Pcr-Cr pathway is as an efficient highway connecting ATP production and consumption sites. Without this highway, cells have to find other ways of ATP and energy transfer, but the efficiency of communication and regulation is lost and energy may be wasted (as in HL-1 cells). Under these conditions, muscle and brain cells degrade into pathological state.

Two important large-scale clinical studies of changes in the Pcr/Cr system in the myocardium of patients with heart failure have been performed [133,134,148]. The first large-scale international study was organized by Ingwall and Allen [148]. In this study, myocardium was sampled from subjects who underwent heart transplant, from subjects maintained in an intensive care unit before heart harvesting, from accident victims, and patients undergoing heart surgery. Since the characteristics of myocardium of potential organ donors differed from those of myocardium of accident victims, data are presented for three groups: failing, donor, and control. MM-Cr and the mitochondrial isoenzyme activities were lower in failing and donor LV, and MB-Crk activity and B-Cr content were higher in failing and donor hearts. Creatine contents were 64±25 and 56±18.6 nmol/mg protein in LV and RV of failing, 96±30 and 110±24 nmol/mg protein in LV and RV of donor, and 131±28 nmol/mg protein in LV of control hearts [148]. Thus, in the failing hearts the total creatine content is significantly decreased, as compared to the control heart. Also, failing hearts showed much lower creatine kinase activity than those of the control [148].

The second important clinical study was performed by Neubauer's group in United Kingdom [133,134]. By using 31P-NMR spectroscopy in combination with imaging for investigation of cardiac muscle energy metabolism in patients, the authors showed that in patients with cardiac disease — dilated cardiomyopathy (DCM) — the decreased
Pcr/ATP ratio (lower than 1.6) is very clear and strong diagnostic index of increased mortality. In the heart of patients with DCM the ATP content remained the same as in healthy control patients, but Pcr decreased by 70% as compared to control. This shows the vital importance of the phosphocreatine-creatine kinase energy transfer network for the cardiac muscle normal function and life [133,134].

**Fig. 13.** Zwitterionic interaction of phosphocreatine with bipolar heads of phospholipid molecules in the membrane surface interphase. Adapted from Saks and Strumia [176] with permission.
It has been found in multiple clinical investigations of biopsy samples of skeletal muscle of patients with heart failure, that it has induced very clear changes of the creatine kinase system in these muscles most probably by decreasing oxygen supply and altering circulation (reviewed in [155,156,161,172]).

Thus, the PCR/ATP ratio is an important diagnostic parameter of heart disease, as is the total creatine content. Low PCr concentrations and low PCR/ATP ratios mean decreased regeneration of ATP by the PCR/CK system in microdomains (compartments) which are critically important for the function of the heart, skeletal muscle and brain. These microdomains are localized in myofibrils, near the sarcolemma and the membrane of sarcoplasmic reticulum in muscle cells and near cellular membrane in brain cells (see Fig. 8). There is a general consensus now among the researchers in muscle and brain energy metabolism that the further challenge and urgent need is to develop better bioprobes to image metabolic microdomains of ATP and functional proteomics to identify physical interactions between key proteins responsible for their formation [133,137,144].

In addition to its important role in supporting regeneration of local ATP pools as a substrate for MM-CK reactions in myofibrils and cellular membranes, the PCR molecule appears to have another very useful property — membrane stabilizing action (Fig. 13). This was revealed in long series of clinical use of extracellular phosphocreatine injection with clear protective effect on ischemic myocardium, and in detailed experimental studies [173–175]. In all these studies extracellular phosphocreatine was used and shown to decrease the ischemic damage of the heart muscle by multiple mechanisms. Among others, there is a clear membrane stabilizing effect of PCR [176] which may be explained by interaction of its zwitterionic molecule carrying positive and negative charges with opposite charges of phospholipid polar heads in the membrane surface interphase (Fig. 13A), resulting in the transition of the mobile domain (fluid phase) of membranes into a structured domain (gel phase), as shown in Fig. 13B, leading to the decrease of the rate of phospholipid degradation into lysophospholipids and lipid peroxidation [176]. Rapid fall of the intracellular PCR pool in hypoxia and ischemia may thus be a significant factor of destabilization of cellular membranes.

In patients with peripheral skeletal muscular diseases, very informative non-invasive diagnostic methods of assessment of the energy state by recording the parameters of the PCR/CK system have been developed due to rapid progress in NMR imaging and spectroscopic technologies [2,133,134,177–180]. Four quantitative parameters of the PCR/CK system which can be measured in patients by 31P and 1H NMR spectroscopy in combination with imaging [122] are: the contents of PCR and ATP and PCR/ATP ratio measured by 31P NMR spectroscopy, the content of creatine measured by 1H NMR spectroscopy [2,154,177–180], the ATP flux through creatine kinase system measured by saturation transfer method [2,154,180], and the kinetics of PCR recovery in skeletal muscle after exercise measured by 31P NMR spectroscopy [2,180]. In combination with the biochemical analysis of biopsy samples taken from skeletal muscles of patients [5,113,181] these methods give an exhaustive diagnostic means for clinical analysis of the energy transfer networks in patients in health and disease [182].

8. General conclusion

Living cells and organisms are thermodynamically opened systems which exchange material and energy with its environment, since they must “avoid the decay into the inert state of equilibrium to keep alive by continually drawing from its environment negative entropy” as it was discovered by Schrödinger [183]. This basic property allows a living system to maintain the “stability of its internal milieu” or homeostasis described already by Claude Bernard [184,185]. Therefore, the cellular life is governed by laws of non-equilibrium irreversible thermodynamics and non-equilibrium steady state kinetics [10,186–190]. A decrease of internal entropy in such systems is achieved by free energy extraction from environment and energy dissipation to realize cellular work [183,188–190]. It is increasingly understood now that in living cells the effective regulation of metabolism is realized through formation of “dissipative metabolic networks” [189] due to complex intracellular interactions within the inhomogeneous intracellular medium. These interactions lead to new system level properties and mechanisms such as intracellular metabolic compartmentation, functional coupling, downward causation including higher level control of gene expression, retrograde response between mitochondria and nuclei etc. which are the topics of studies in Molecular System Bioenergetics [10,67], part of Systems Biology [65–67].

Results reviewed in this article show the importance of structural and functional organization of intracellular phosphotransfer networks interconnecting ATP-utilization and ATP regeneration processes into intracellular energy units (ICEU). The role of the ICEUs is not reduced only to the high efficiency of coordination of energy metabolism. Their role is more fundamental: in conformity with the theory of dissipative metabolic structures [189,190], formation of the ICEUs helps to extract Gibbs free energy and negentropy from the environment. ICEU can be seen as a “dissipative metabolic network” which functions in the nonequilibrium state made up of the dissipative enzymatic sub-networks (glycolysis, the Krebs cycle, fatty-acid’s oxidation, the electrons transport chain, the shuttles creatine kinase/phosphocreatine, malate/aspartate, etc.) structured and connected together by flows and regulating signals. Association of various enzymes within big multienzyme complexes allows the direct transfer of intermediate metabolites (vesselary ligand conduction). One of such complexes is Mitochondrial Interactusome which regulates the interaction between mitochondrial cycles of adenine nucleotides and PCR/Cr cycles in the cytoplasm of the heart, skeletal muscle and brain cells. Mitochondrial Interactusome and PCR pathway of intracellular energy transfer explain well the metabolic aspects of Frank-Starling law of the heart and classical observation of Belitser and Tsymbalova on effective coupling of PCR production and oxidative phosphorylation in muscles. Changes in Mitochondrial Interactusome lead to severe pathology and may contribute in the mechanism of Warburg effect in cancer cells. Systemic analysis of changes in phosphotransfer networks helps to explain many pathogenic mechanisms in numerous diseases.

Acknowledgements

This work was supported by INSERM, France, by Agence Nationale de la Recherche, project ANR-07-BLAN-0085-01 France, by grants of Estonian Science Foundation nos. 7823 and 6142, and by grants SF 0182549AasG and SF 0180114As08 from the Estonian Ministry of Education and Research. The authors thank Peeter Sik and Tia Anmann from Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Kalju Paju, Ehte Orlova Marju Gruno, Mart Roosimaa from Department of Pathophysiology, Faculty of Medicine, University of Tartu, and Andres Piirsoo from Department of Human Biology and Genetics, Faculty of Medicine, University of Tartu, Tartu, Estonia, for their active participation in the investigations. Finally, the authors thank the anonymous reviewer of this work for carefully reading this manuscript twice and for his constructive criticism that helped very much to improve the quality and clarity of the presentation, and Edmund Sherwood, Goldsmiths College, University of London, UK, for correcting the English of this text.

References

DISSEMINATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON NATURAL AND EXACT SCIENCES


33. **Merike Peld.** Substituted Apatites as Sorbents for Heavy Metals. 2005.

34. **Vitali Syritski.** Study of Synthesis and Redox Switching of Polypyrrole and Poly(3,4-ethylenedioxythiophene) by Using in-situ Techniques. 2004.


41. **Maksim Säkki.** Intermittency and Long-Range Structurization of Heart Rate. 2005.


47. **Maria Kulp.** Capillary Electrophoretic Monitoring of Biochemical Reaction Kinetics. 2006.

49. **Lyudmila Chekulayeva.** Photosensitized Inactivation of Tumor Cells by Porphyrins and Chlorins. 2006.
50. **Merle Uudsemaa.** Quantum-Chemical Modeling of Solvated First Row Transition Metal Ions. 2006.
52. **Angela Ivask.** Luminescent Recombinant Sensor Bacteria for the Analysis of Bioavailable Heavy Metals. 2006.
55. **Ildar Nisamedtínov.** Application of $^{13}$C and Fluorescence Labeling in Metabolic Studies of *Saccharomyces* spp. 2006.
60. **Julia Kois.** Electrochemical Deposition of CuInSe$_2$ Thin Films for Photovoltaic Applications. 2006.
61. **Ilona Oja Açik.** Sol-Gel Deposition of Titanium Dioxide Films. 2007.
63. **Katrin Trummal.** Purification, Characterization and Specificity Studies of Metalloproteinases from *Vipera lebetina* Snake Venom. 2007.
66. **Maria Borissova.** Capillary Electrophoresis on Alkylimidazolium Salts. 2007.
72. **Vilja Mardla.** Inhibition of Platelet Aggregation with Combination of Antiplatelet Agents. 2008.
82. Anna Menaker. Electrosynthesized Conducting Polymers, Polypyrrole and Poly(3,4-ethylenedioxythiophene), for Molecular Imprinting. 2009.
103. Kristi Timmo. Formation of Properties of CuInSe2 and Cu2ZnSn(S,Se)4 Monograin Powders Synthesized in Molten KI. 2011.
106. Ove Pärn. Sea Ice Deformation Events in the Gulf of Finland and This Impact on Shipping. 2011.
125. Indrek Reile. 3-Alkylecyclopentane-1,2-Diones in Asymmetric Oxidation and Alkylation Reactions. 2012.
160. Irina Stulova. The Effects of Milk Composition and Treatment on the Growth of Lactic Acid Bacteria. 2013.