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INTEGRATED AND ORGANIZED CELLULAR BIOENERGETIC SYSTEMS IN HEART AND BRAIN

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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 degree or examination.

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ABBREVIATIONS

ADP	—	adenosine diphosphate
AK	—	adenylate kinase
AMP	—	Adenosine monophosphate
ANT	—	adenine nucleotide translocase
ATP	—	adenosine triphosphate
BB-CK	—	brain type creatine kinase
BSA	—	bovine serum albumin
CCCP	—	carbonyl-cyanide-m-chlorophenyl-hydrazone
CK	—	creatine kinase
Cr	—	creatine
DTT	—	dithiothreitol
EGTA	—	ethyleneglycol-bis(ß-amino- ethyl ether)-*N,N,N',N`*-tetra acetic acid
$FADH_2$	—	1,5-dihydro-flavin adenine dinucleotide
FCCP	—	carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone
HEPES	—	*N*-2-hydroxyethyl-piperazine-*N`*-2-ethanesulfonic acid
ICEU	—	intracellular energetic unit
K_m	—	Michaelis constant
LDH	—	lactatedehydrogenase
MB-CK	—	hybrid type creatine kinase
MES	—	2-(*N*-morpholino) ethanesulfonic acid
MIM	—	mitochondrial inner membrane
MM-CK	—	muscle type creatine kinase
MOM	—	mitochondrial outer membrane
MtCK	—	mitochondrial creatine kinase
NAD^+	—	nicotinamide-adenine dinucleotide
NADH	—	nicotinamide adenine dinucleotide
NADP	—	nicotinamide adenine dinucleotide phosphate
NCX	—	Na^+/Ca^{2+} exchanger
PCr	—	phosphocreatine
PEP	—	phosphoenolpyruvate
PK	—	pyruvate kinase
PLB	—	phospholamban
PMF	—	proton motive force
PTP	—	permeability transition pore
Rhod-2	—	red fluorescent dye
sMtCK	—	sarcomeric mitochondrial creatine kinase
SR	—	sarcoplasmic reticulum
TCA	—	tricarboxylic acid cycle
TMRE	—	tetramethylrhodamine ethyl ester
TMRM	—	tetramethylrhodamine methyl ester
U	—	international unit
uMtCK	—	ubiquitous mitochondrial creatine kinase
VDAC	—	voltage dependent anion channel

INTRODUCTION

The awareness about importance of cellular structure has remarkably risen within the last decade. The interior of the cell is no longer considered as a well mixed bag of enzymes and other cellular components, and the intracellular compartments are considered to be more important than just being separated by internal membranes volumes. Cytoplasm is nowadays considered as a system of microcompartments with highly organized cytoskeleton, diffusion restrictions and transfer systems for different molecules (Weiss and Korge, 2001). Regular arrangement of mitochondria with very high diffusion restrictions for exogenous ADP, and more favored transport conditions for endogenous ADP in regulation of respiration in mitochondria is speaking clearly about high degree of compartmentation in the cardiac cells. This has led to the hypothesis that mitochondria and ATPases are compartmentalized into functional complexes (i.e. intracellular energetic units, ICEU) (Saks *et al.,* 2001). According to this hypothesis a complex cellular organization is regulating energy metabolism in cardiac cells. Therefore, more information is needed about relations between cellular organization and mechanism of respiration regulation. The role of cellular structure and internal organization in regulation of mitochondrial respiration in cardiac cells is investigated in this study.

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1. REVIEW OF LITERATURE

1.1. Structure of cardiac cells

There are two categories and three types of muscles: a) skeletal muscle and cardiac muscle - both striated muscles with sarcomeres, and b) smooth muscle without sarcomeres. Skeletal muscles are anchored to the bone and are used for skeletal movement and maintaining posture. Smooth muscles are found in internal organs (digestive and reproductive tracts) and in the blood vessels. Cardiac muscle is very specialized for heart function, for continuous work with high need for energy. The structure of cardiac muscle should support its function: high capacity to produce ATP as source of energy, and respond quickly to the need for energy depending on the workload of the heart. Therefore, cardiac muscle is an oxidative muscle, dense with capillaries and rich in mitochondria (35-40% of cell volume) and myoglobin, giving characteristic red color for this muscle tissue (Opie, 1998).

Cardiac muscle cells, also called cardiomyocytes, are 10-25 μm in diameter and ~50-150 μm in length from ventricle and 10 μm in diameter and 20 μm in length from atrium. Cardiomyocytes are very small compared to skeletal muscle, which are 80-100 μm in diameter and the length is determined by the length of the muscle (Opie, 1998).

Cardiac muscle cells are branched and connected to each other through intercalated discs (Figure 1) to form uniformly functioning organ (working as syncytium), where cytoplasm of adjacent cells is connected by cap junctions which allows excitation signal to travel rapidly from cell to cell throughout the heart and synchronize contraction of cardiac cells. This direct connection is important for conductance of ionic currents in normal cardiac function but also in development (Gallicano *et al.,* 1998) and is achieved at the intercalated discs by adherens junctions and desmosomes (Angst *et al.,* 1997).

Structure of cardiac cells is very regular and well organized, with parallel rows of myofibrils and mitochondria (Figure 1). Framework for structure of cardiac cells is formed by cytoskeleton and sarcomeric structure. The unique cytoarchitecture of cardiomyocytes arises by complex interactions of different proteins: filamentous structures of the cytoskeleton (microfilaments, intermediate filaments and microtubules), sarcomeric skeleton (α-actinin, titin, C-protein, myomesin, M-protein), membrane-associated proteins (dystrophin, spectrin, talin, vinculin, ankyrin) and proteins of the intercalated disc (desmoplakin, N-cadherin, catenins, vinculin, connexin etc.) (Kostin *et al.,* 1998). These proteins contribute to cell shape, morphological integrity and mechanical resistance by anchoring different subcellular structures, such as mitochondria, the sarcoplasmic reticulum (SR), nuclei, sarcolemma and contractile units. Multiple ultrastructural studies done with electron microscopy show connections between mitochondria and cytoskeleton elements (Lockard and Bloom, 1993; Penman, 1995; Rappaport *et al.,* 1998). Beside the structure of the cardiac muscle, the cytoskeleton also contributes into the force transmission, the contraction, the signaling and the intracellular transport.

The single contractile units in the muscle cells are the sarcomeres which are connected to each other by Z-lines forming myofibrils. The myofilaments are the contractile machinery of the muscle cells, which are responsible for transducing the chemical energy into mechanical energy and work. They occupy 45-60% of the cell volume in mammalian ventricle (Bers, 2001). Myofilaments are composed of the thick (myosin) in A-bands and thin (actin)

filaments in I-bands (Figure 2). The thick myosin filaments are ~ 1.6 µm long and 15 nm thick. Each thick filament is composed of ~300 myosin molecules. Myosin heads (known as myosin ATPase) protrude from the long axis after every 14.3 nm, with the protrusion angle rotating 120^{o} each point. Thin filaments are consisting of actin, tropomyosin and troponin. Rod-like tropomyosin molecules are forming polymers which stabilize the thin filaments. Myofilaments are anchored in Z-lines: actin filaments are connected directly and myosin filaments are secured via the giant elastic protein titin (Squire, 1997).

The contraction of the cardiac muscle is triggered by internal pacemaker cells independently from a nerve impulse, in contrast to the skeletal muscle, which contracts only when stimulated via a nerve impulse. The general scheme of contraction (through formation of actomyosin complexes in contraction cycle) is similar in cardiac and skeletal muscles, but the excitation-contraction coupling is very different. Skeletal muscles contraction depends mostly upon Ca^{2+} released from SR with quantitatively insignificant Ca^{2+} entry across the sarcolemma. Cardiac contraction, on the other hand, depends on both, Ca^{2+} entry across sarcoplasma and Ca^{2+} release from the SR (Bers, 2001). Cardiac SR is sparse and less rigidly organized with much larger diameters of T-tubules with mean diameter 200-300 nm (Bers, 2001; Brette and Orchard, 2003) *vs.* 30-40 nm in skeletal muscle (Bers, 2001). T-system is necessary for the rapid propagation of the excitation impulse throughout the muscle fiber in both skeletal and heart muscles (Sommer, 1995). T-tubules occur predominantly (60%) at the end of each sarcomere (at the Z line) in ventricular myocytes (Figure 1).

Cardiomyocytes are bound by an outer membrane, the sarcolemma, which is high in cholesterol content. This is used in experimental studies for permeabilization of the sarcolemma by detergents (digitonin or saponin). Cells stay intact after permeabilization as mitochondrial membranes have notably low content of cholesterol. The surface of the sarcolemma is physically continuous with the membrane of the T-tubules, which have many transverse and longitudinal components (Soeller and Cannell, 1999).

Cardiac muscle cells are surrounded by an extracellular matrix (ECM) which is composed of glycoproteins, collagen and proteoglycans and is important in cell-cell interactions. Extracellular matrix of neighbor cardiomyocytes is connected by specific cell surface adhesion molecules known as integrins. The latter link mechanically the cytoskeleton to the extracellular matrix and are also involved in mechanotransduction. ECM plays a vital role in force transmission throughout the myocardium, but it is also essential in cardiac development, growth, and responses to patophysiological signals through integrin-mediated cell-ECM interactions (Ross and Borg, 2001).

1.2. Consumption of energy

Under normal conditions, the majority of adenosine triphosphate (ATP) formation (>95%) in the heart comes from oxidative phosphorylation in the mitochondria by aerobic oxidation of fatty acids and carbohydrate substrates. The remaining ATP is derived from glycolysis and GTP formation in the citric acid cycle. The majority of ATP is consumed by ATPases in myofilaments (60-70%) and the remaining (30–40%) is utilized by Ca^{2+}-ATPase (Ca^{2+}-pump) in sarcoplasmic reticulum for regulation of intracellular Ca^{2+} concentration (Opie, 1998; Stanley *et al.,* 2005; Taegtmeyer *et al.,* 2005). A minority of the is utilized by active transport of different ions and substrates, anabolic reactions and cellular signaling.

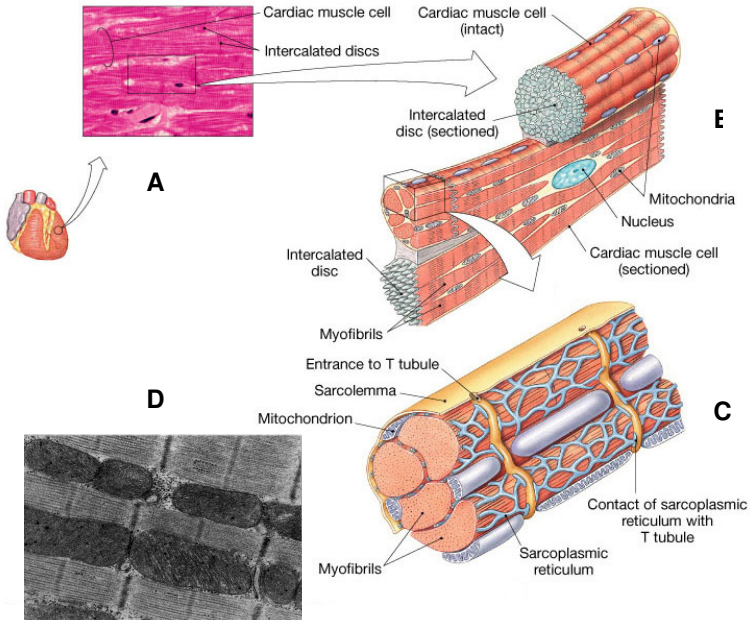

Figure 1 Ultrastructure of cardiac cell. A: Cardiac muscle; B: Cardiac muscle cell; C: magnification of B; D: Electron microscopy of cardiac cell.

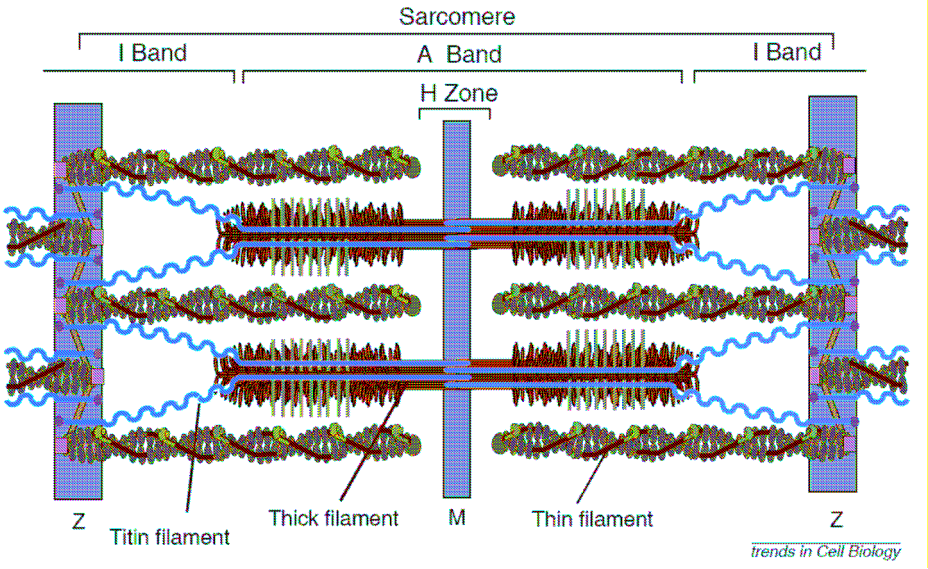

Figure 2 Major components of a cardiac muscle sarcomere: a single contractile unit. Actin (green), myosin (red). Rod-like tropomyosin molecules (black lines). Thin filaments in muscle sarcomeres are anchored at the Z-disk by the cross-linking protein α-actinin (gold) and are capped by CapZ (pink squares). The thin-filament pointed ends terminate within the A band, are capped by tropomodulin (bright red). Myosin-binding-protein C (yellow transverse lines) (from Gregorio and Antin, 2000 with permission)

1.2.1. Contraction cycle of cardiomyocytes

Contraction is activated spontaneously by pacemaker cells and electrical signal spreads through the heart quickly from cell to cell and each cardiac cell is activated on each heart beat. Contraction is triggered by Ca^{2+}-induced Ca^{2+} release from the SR and thus elevation of intracellular Ca^{2+} concentration. The released Ca^{2+} binds to troponin C on the thin filaments and causes conformational changes. Contraction is created by interactions between myosin S1 heads (M) and actin filaments (A) (Geeves and Holmes, 1999) after activation of thin filaments in a Ca^{2+}-dependent manner. Force develops as a result of the interaction of myosin heads with the thin filament according to the sliding filament theory (Huxley, 1969; Huxley and Simmons, 1971), and of the formation of crossbridges and conformational changes in myosin heads (crossbridge cycle). Binding of myosin heads to actin increases affinity of Ca^{2+} binding to troponin C (Fuchs, 1995). The energy for conformational changes that drive force generation is provided by ATP hydrolysis. Therefore, myosin heads are transducing the chemical energy (ATP) into mechanical force with production of ADP and P_i. This can be formally represented as a chemical reaction in the form:

$$A + M + ATP \rightarrow A + M + ADP + Pi + force \quad (1)$$

Crossbridge cycle is shown with all reactants and products in Figure 3A and with structural changes in eight steps in Figure 3B. Although the cycle is the same for skeletal and cardiac muscles, the rate constants controlling crossbridge intermediate transitions differ. The ratelimiting step is the release of products of ATP hydrolysis. The hydrolysis of ATP to ADP and P_i requires magnesium ions (Gordon *et al.,* 2001). Step 1, is a very rapid and irreversible binding of ATP to myosin. Step 2, is a rapid detachment of actin from the actin-myosin-ATP complex (A~M•ATP) caused by an opening between myosin's upper and lower 50-kDa regions (or opening of jaws). Step 3, is a "flexing" or bending of the myosin neck region. At step 4, myosin binds weakly to the actin at a high rate — in the absence of Ca^{2+} access of the myosin heads to the strong binding sites on actin is blocked by troponin. At step 5, strong binding of myosin to actin is dependent on Ca^{2+} and troponin position and is associated with movement of the upper and lower 50-kDa subdomains toward each other (or closing the jaws). At step 6, the power stroke that, in isometric muscle, stretches an elastic element (black) by some 10 nm and produces a force of ~2pN/cross-bridge (Molloy *et al.,* 1995), but causes the thick and thin filaments to slide over each other in nonisometric conditions. Step 7 is the rate-limiting step for the crossbridge cycle, with irreversible isomerization. Finally, ADP is released from $A{\cdot}M^{f}{\cdot}ADP$ in the reversible step 8 to form the rigor state. Crossbridges attach and exert force constantly during steps 7, 8, and 1 during isometric contraction, and force drops to zero when the cross-bridges detach in step 2 (Gordon *et al.,* 2001). The cycle can continue until $[Ca^{2+}]$ declines, thereby stopping myofilament interaction or until ATP is depleted (Bers, 2001).

Cardiac muscle is a slow-twitch muscle, which means longer time to peak contraction and slower rate of contraction, lower ATPase activity and slower rate of ATP breakdown compared to fast-twitch skeletal muscles which have higher ATPase activity and therefore catalyse a more rapid breakdown of ATP.

The major difference between cardiac and skeletal muscles, besides activation of contraction, is the modulation of the extent of thin filament activation. Force development must be controlled mainly at the cellular level, because each cardiac cell is activated on each

beat and each cell must be able to undergo the full dynamic range of cardiac output (Gordon *et al.,* 2001). The length-tension relationship in muscle, which states that force in any sarcomere length is determined by the degree of the thick and thin filaments overlap (Gordon, 1966), only explains ~20 % of the classic Frank-Starling law in heart (Bers, 2001). According to this law, an increased diastolic volume leads to an increased cytosolic contraction — length dependent activation. Thus, when the cardiac muscle is stretched there is an immediate increase in contractility. This fundamental principle of heart is explained by increase in cell or sarcomere length that leads to an increase in force due to the rise in Ca^{2+} sensitivity and an increase in the probability of crossbridge formation (Robinson *et al.,* 2002).

1.2.2. Ca^{2+} cycle

Intracellular [Ca^{2+}] is strictly regulated by Ca^{2+}-ATPase pumps in SR and sarcolemma, and mitochondrial uniporter, because Ca^{2+} is important mediator in intracellular signaling (Jacobson and Duchen, 2004) and it regulates contraction-relaxation cycle, metabolism (activates dehydrogenases in the tricarboxylic acid cycle), protein synthesis, gene expression and apoptosis (Hansford and Zorov, 1998; Bernardi, 1999; Rizzuto *et al.,* 2000).

The cytoplasmic free Ca^{2+} concentration in diastolic (resting) muscle is about 20–50 nM, and the systolic peak values are up to 10^{-5} M, depending on the contractile state of the myocardium (Opie, 1998). The free Ca^{2+} concentration in the extracellular space or in the lumen of SR are usually millimolar. The large Ca^{2+} gradients across cellular boundaries are established and maintained by powerful Ca^{2+} pumps located in the cell surface membranes, and in the SR (MacLennan *et al.,* 1997; Guerini and Carafoli, 1999; Philipson and Nicoll, 2000), with contributions by the mitochondrial Ca^{2+} uniporter (Rizzuto *et al.,* 2000).

The calcium-excitation cycle is shown in Figure 4. Ca^{2+} enters and leaves the cell through sarcolemma by ion channels (L–type Ca^{2+} channels), which have a very high density in T-tubules (Wibo *et al,.* 1991), by Ca^{2+}-pumps on sarcolemma and through intercalated discs, where the cells are closely apposed end to end. L–type Ca^{2+} channels are voltage dependent Ca^{2+}channels, which respond to changes in the membrane potential (Bezanilla, 2000; Catterall, 2000) and control opening of the ryanodine receptor proteins (RyR channel), either by direct interaction with RyR or through contacts with other proteins. Trans-sarcolemmal Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release from SR (Fabiato, 1992) play a major role in the rise of $[Ca^{2+}]_i$, which activates contraction in myofilaments by Ca^{2+}-dependent manner. There may be also some entry of Ca^{2+} via Na^+/Ca^{2+} exchanger (sarcolemmal Na^+/Ca^{2+}-ATPase pump, NCX), which catalyzes the electrogenic exchange of 3 Na^+ for 1 Ca^{2+} across the surface membrane (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000). Cyclic variations of cytosolic $Ca^{2+}$are called calcium transients. There is evidence that Ca^{2+} transients does not change during alteration of the heart workload (Shimizu *et al.,* 2002).

Ca^{2+} must be removed from the cytoplasm for relaxation. Ca^{2+} is removed from the myofilaments by the SR Ca-ATPase pump (SERCA), which is modulated by phospholamban (PLB), sarcolemmal Ca^{2+}-ATPase pump, NCX and mitochondrial uniporter (Bers, 2000). All these Ca^{2+} transport systems are in direct competition for cytoplasmic [Ca^{2+}]. In the rat ventricle, the SERCA removes 92% of the Ca^{2+} from the cytosol, whereas the NCX removes 7%, with only ~1% for the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter (Hove-Madsen and Bers, 1993).

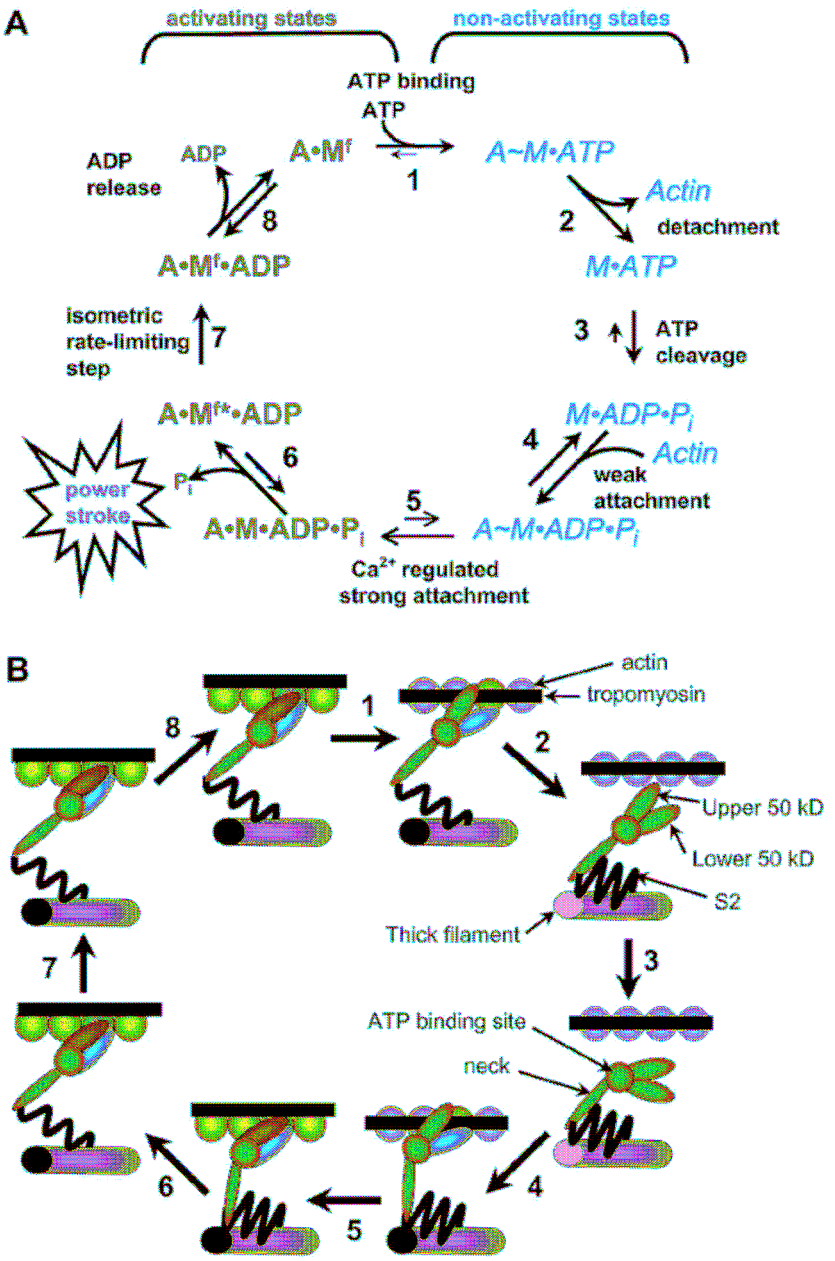

Figure 3 Contraction cycle in muscle cells. A: Reaction steps in cross-bridge cycle. Strong binding is designated by "·"and weak binding by "~". A, actin; M, myosin; P_i, inorganic phosphate; $A{\cdot}M^f$, f is a cross-bridge exerting force. B: Cross-bridge cycle with structural changes. Non-interacting cross-bridge-actin pairs are shown as gray actin and green myosin, weak interactions as yellow actin and light blue myosin, and strong interactions as green actin and red myosin (from Gordon *et al.,* 2001 with permission)

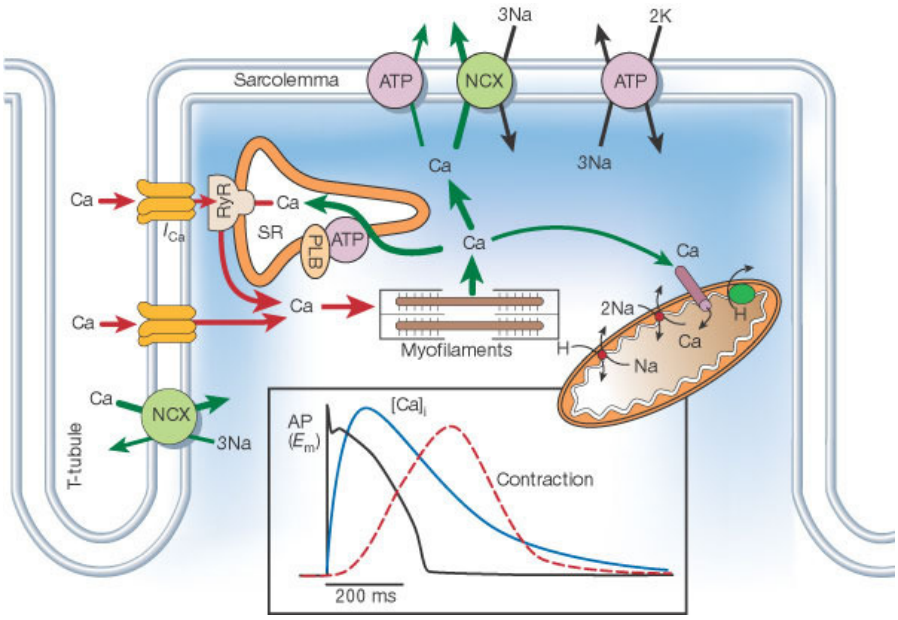

Figure 4 Cardiac excitation-contraction-coupling in a ventricular myocyte. Electrical excitation at the sarcolemmal membrane activates voltage-gated Ca^{2+}channels, and the resulting Ca^{2+} entry activates Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs), resulting in contractile element activation. NCX, Na^+/Ca^{2+} exchanger; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum. Graph shows the time course of an action potential, Ca^{2+} transient and contraction (from Bers, 2002 with permission)

1.3. Free energy conversion in mitochondria

The main function of the mitochondria is the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by the process of oxidative phosphorylation. Mitochondria are the centers for free energy conversion in cardiac muscle, as they contribute 90% of the energy for the cardiac cell use. Cardiac muscle achieves the highest sustained metabolic rates of any tissue in the body (heart mitochondria consume 50 times more oxygen than liver mitochondria (Perkins and Frey, 2000)), cardiac muscle cells are very rich in mitochondria, ~35% of the ventricular volume is occupied by mitochondria (Bers, 2001). High content of mitochondria gives increased surface area for production of ATP; the surface area in folded inner mitochondrial membrane has been estimated to be 20 $\mu m^2/\mu m^3$ of cardiomyocyte volume (Bers, 2001).

Although a dominant role for the mitochondria is the production of ATP, mitochondria play an important role also in many other metabolic tasks such as: fatty acid oxidation, intracellular calcium homeostasis, regulation of cellular redox state, osmotic regulation, signaling events, heat production during electron transport in respiratory chain and participation in apoptosis. The mitochondria have similar functions in all eukaryotic cells, although the structure of mitochondria is very varied in different species and is dependent on the function of cells.

1.3.1. Structure of mitochondria

The localization of mitochondria is very tissue specific and is considered to play a central role in the regulation of respiration in cells (Ogata and Yamasaki, 1993). Mitochondria are very regularly arranged in cardiomyocytes, in parallel rows between bundles of myofibrils, forming crystal-like structure at the level of A-bands of sarcomeres in intact cardiomyocytes (Vendelin, *et al.,* 2005) and also form structural and functional complexes with sarcoplasmic reticulum and sarcomere (Saks *et al.,* 2001; Seppet *et al.,* 2001; Kaasik *et al.,* 2001).

The mitochondria are rod-shaped organelles with two membranes, inner and outer mitochondrial membrane. The mitochondrial inner membrane (MIM) separates the extremely protein-dense matrix space from the intermembrane space. The inner membrane forms protuberances into the interior known as cristae. The mitochondrial cristae of the inner membrane are the sites of ATP production. The intermembrane space is the area between the inner and outer membranes. The mitochondrial outer membrane (MOM) is smooth and works as a selective barrier, as it contains porin channels, which are permeable for ions and molecules up to 5 kDa. Larger molecules are able to traverse MOM only by active transport. The MIM is highly impermeable. Almost all ions and molecules (such as pyruvate, fatty acids and amino acids etc.) require special transporters to enter or exit mitochondrial matrix. The latter is a very concentrated aqueous solution of intermediates of metabolism and hundreds of enzymes for the citric acid cycle, amino acid oxidation and fatty acid β-oxidation. It also contains multiple copies of mitochondrial DNA that encodes proteins for the respiratory chain, the ATP synthase and proteins for their translation.

There are two models of the structure of mitochondria: baffle (Palade, 1952) and cristae junction model (Sjostrand, 1953) (Figure 5). According to baffle model, the MIM forms infoldings with large openings connecting the intra cristal space to the membrane space (Figure 5a). Baffle model was developed on the basis of the transmission electron microscope data, but novel applications of microscopy, such as high resolution scanning electron microscopy and electron microscope tomography, which have recently provided new insights into mitochondrial structure.

Baffle model was developed on the basis of the transmission electron microscope data, but novel applications of microscopy, such as high resolution scanning electron microscopy and electron microscope tomography have recently provided new insights into mitochondrial structure. Recent studies have shown that there is no continuity between cristae and peripheral membranes and the cristal and inner boundary membranes are joined by a limited number of discrete sites called crista junctions (narrow tubular openings with diameter of ~30 nm) (Figure 5b), which may be altered by matrix volume and respiratory activity (Lea *et al.,* 1994; Perkins and Frey, 2000). In the heart the subsarcolemmal mitochondria possess mainly lamelliform cristae (77%), whereas the cristae in the interfibrillar mitochondria are mainly tubular (55%) or a mixture of tubular and lamelliform (24%) (Riva *et al.,* 2005). Observation that striated muscles contain crista tubes and lamellae in varying proportions, led to the conclusion that cristae conformation is a direct consequence of the specialized function of the tissue (Lea *et al.,* 1994).

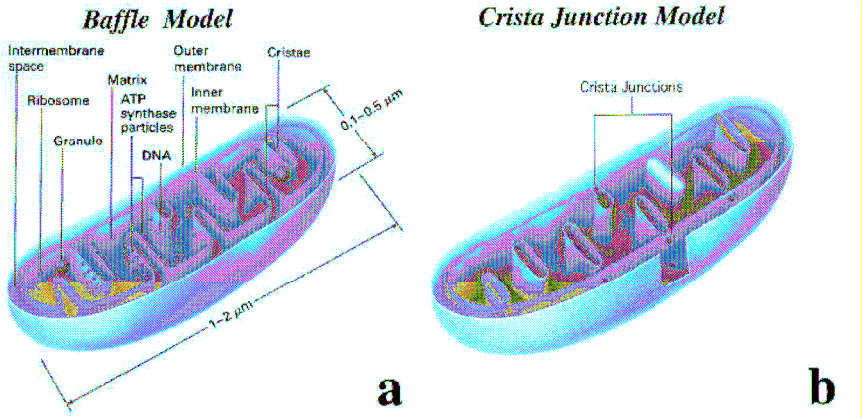

Figure 5 Models of mitochondrial membrane structures. (a) Infolding or "baffle" model. (b) Crista junction model (from Perkins and Frey, 2000 with permission)

1.3.2. Respiratory chain

Fatty acids, lactate and glucose are the main are main energy substrates in cardiac muscle. The energy substrates are oxidized by glycolysis (localized in the cytosol) or β-oxidation (localized in the mitochondrial matrix). Products of oxidation are used in the form of acetylcoenzyme A (acetyl-CoA) by the enzymes of tricarbocylic acid cycle (TCA) in the mitochondrial matrix (except succinate dehydrogenase, which is bound to the MIM). The TCA cycle oxidizes the acetyl-CoA to carbon dioxide and in the process produces reduced cofactors, nicotinamide adenine dinucleotide and 1,5-dihydro-flavin adenine dinucleotide (three molecules of NADH and one molecule of $FADH_2$ respectively), that are sources of electrons for the electron transport chain, and a molecule of guanosine triphosphate (GTP) which is converted to ATP.

The energy from NADH and $FADH_2$ is transferred to oxygen (O_2) in several steps via the electron transport chain (Figure 6) that consists of four respiratory enzyme complexes (complex I, II, III and IV) arranged a specific orientation inside the MIM. These enzyme complexes perform the electron transfer that releases the energy to pump protons (H^+) into the intermembrane space of mitochondria. NADH carries electrons to the complex I (NADH dehydrogenase) and $FADH_2$ directly to the complex II (succinate dehydrogenase). Three of respiratory enzyme complexes (complex I, complex III – cytochrome *c* reductase, complex IV – cytochrome c oxidase) are working as proton pumps establishing the electrochemical potential gradient (the proton motive force) across inner mitochondrial membrane (Figure 6). The proton motive force (PMF) consists of an electrical ($\Delta\psi$) (membrane potential) and chemical part (ΔpHm).

1.3.3. Oxidative phosphorylation

The oxidative phosphorylation couples oxidation of NADH and succinate by respiratory chain (the electron transport chain) and synthesis of ATP by ATP synthase on the inner mitochondrial membrane. The protons which are pumped by respiratory chain to the intermembrane space may return into the mitochondrial matrix via the ATP synthase (F_oF_1-

ATP synthase) complex and the potential energy is used to synthesize ATP from ADP and inorganic phosphate (P_i).

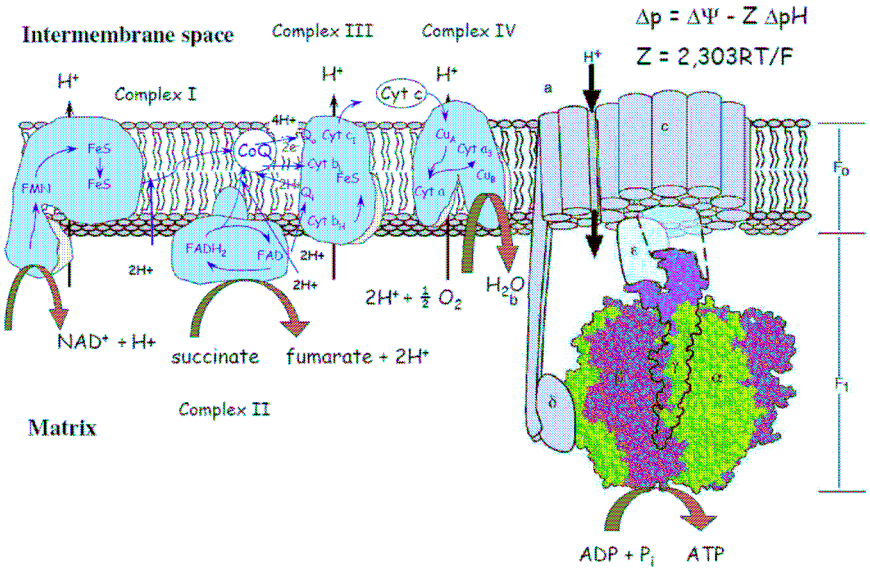

Figure 6 Oxidative phosphorylation. The respiratory chain (consisting of complexes I, II , III and IV) and ATP synthase (consisting of two major part: F_o is embedded in the membrane and F_1 is above membrane) (Modified from: Wang and Oster, 1998)

The understanding of ATP synthesis by coupling of the proton motive force with phosphorylation is based on the chemiosmotic theory introduced by Mitchell (Mitchell, 1961). This theory explains the chemiosmotic coupling of two reactions respiration and phosphorylation — oxidative phosphorylation in mitochondria.

Intact inner mitochondrial membrane is relatively impermeable to the back flow of the protons. However, under certain conditions, protons may re-enter into the mitochondrial matrix without contributing to ATP synthesis. This process is known as the proton leak or the mitochondrial uncoupling. Uncoupling agents (dinitrophenol, CCCP - carbonyl cyanide m-chloro phenyl hydrazone, FCCP - carbonylcyanide-p-trifluorometoxy-phenylhydrazone and fatty acids) abolish the obligatory linkage between the respiratory chain and the phosphorylation system and thus facilitate the diffusion of protons into the matrix. This causes a decrease in the proton gradient (Δp) which leads to a waste of energy and increases thermogenesis (Kadenbach, 2003).

1.3.4. The mitochondrial adenosine triphosphate synthase

The mitochondrial adenosine triphosphate synthase (ATP synthase, also called F_oF_1ATPase) is a membrane-bound multisubunit enzyme that couples the proton motive force across the inner mitochondrial membrane to the synthesis or hydrolysis of ATP in the matrix (Boyer, 1997; Walker, 1998). The general structure of F_oF_1ATPase is shown in Figure 6. This enzyme has two major structural parts F_o and F_1. The F_o is embedded in the membrane as an integral part of the inner mitochondrial membrane and mediates proton translocation into the mitochondrial matrix through the proton channel. The F_1 protrudes from the inner

mitochondrial membrane into the matrix and has three catalytic sites of the ATP synthesis. F_o and F_1 are working like rotor and stator assembly, respectively. The rotor consists of 12 c-subunits arranged in a ring and connected to the γ- and ε-subunits that form the central `shaft' (Wang and Oster, 1998). The stator portion consists of the catalytic sites contained in the $\alpha_3\beta_3$ hexamer, together with subunits a, b_2 and δ (Walker, 1998; Collinson *et al.,* 1996).

The rotation is produced in the F_o between the ring of c-subunits and the a-subunit, when the central shaft and with it a asymmetric, rigid and long γ –subunit in the interior of $\alpha_3\beta_3$ complex is turned clockwise by the protonmotive force (Wang and Oster, 1998). The rotation is counterclockwise to pump protons when hydrolyzing ATP. This γ –subunit is in contact with β subunits of F_1 and its rotation drives the successive conformational changes of catalytic domains of β subunits of F_1 to induce the change of its affinity to ATP (binding-change mechanism). Hydrolysis of ATP induces reverse conformational changes, and thus reverses rotation of the γ –subunit and connected to it 12 subunits c of F_o (Noji *et al.,* 1997). The central stalk rotates about 50-100 times per second.

The rotatory mechanism of ATP synthesis by F_oF_1ATPase was proposed by Boyer (Boyer 1997). The asymmetric central rotor of ATP synthase has the ability to rotate 360° relative to the stator. Boyer proposed a `binding-change mechanism´ where three catalytic sites (Figure 7) pass sequentially through three different conformations linked to subunit rotation (Boyer, 1993, 1997). Each catalytic site has different affinities for nucleotides at any moment; each undergoes conformational transitions that lead to the sequence of substrate binding, ATP synthesis and ATP release. According to the binding-change mechanism filling of one or two sites is not enough for rotation, and only the filling of the third catalytic site brings about the conformational changes and catalytic cooperativity that are critical for operation of the mechanoenzymatic mechanism (Weber and Senior, 2003). At low ATP concentrations the rotary motor rotates in discrete 120° steps. Each 120° step consists of roughly 90° and 30° substeps, each taking only a fraction of a millisecond (Yasuda *et al.,* 2001). Although the binding-change mechanism is widely accepted, the mechanism of stepping is unknown. Direct observation of the rotation of the γ subunit of F_1 has provided strong experimental support for the binding change mechanism (Yasuda *et al*., 2001).

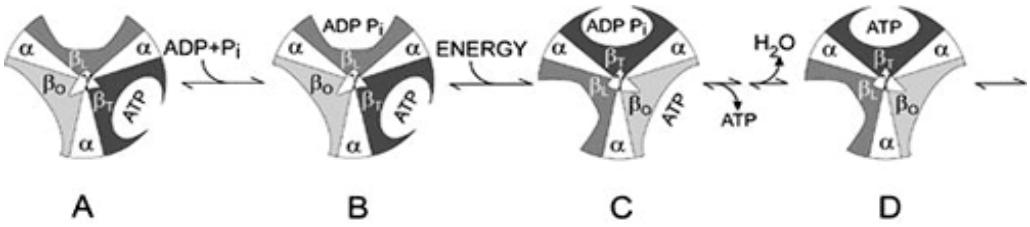

Figure 7 The mechanism of ATP synthesis: "Binding-Change Mechanism". The α and β subunits are alternating at four different stages of ATP synthesis. The asymmetrical γ subunit causes changes in the structure of the β subunits: open β_O (light grey sector), loose β_L (grey sector) and tight β_T (black sector). One full rotation of rotor produces 3 ATP.

1.3.5. Respiratory regulation by ADP

Transfer of electrons in respiratory chain occurs only with the rephosphorylation of ADP. Thus the rate of respiration depends on the proton motive force (PMF) and the presence of

ADP and phosphate in mitochondria. This phenomenon is termed respiratory control by ADP and implies the fact that the oxidation of NADH and $FADH_2$ is coupled to the transport of H^+ across the inner membrane. The existence of respiratory control and the uncoupling effect of the dinitrophenol on respiration of isolated mitochondria have been experimentally demonstrated by Lardy and Wellmann (Lardy and Wellman, 1952).

The efficiency of the oxidative phosphorylation is measured as a P/O ratio, the amount of inorganic phosphate incorporated into ATP per amount of consumed oxygen molecule. The oxidation of NADH, $FADH_2$ and ascorbate by O_2 are associated with the synthesis of three, two and one ATP respectively. The theoretical maximal P/O ratio is 2.5-3 for NAD-linked substrates and 1.5-2 for succinate (Lee *et al.,* 1999). Uncouplers of oxidative phosphorylation also decrease the P/O ratio, because they degrade the proton gradient (Terada 1990).

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The regulation of oxidative phosphorylation through a negative feedback of ADP was discovered *in vitro* in isolated mitochondria by Chance and Williams (Chance and Williams, 1955). The main characteristic for mitochondria is the dependence of the oxygen consumption rate on free ADP concentration. The rate of respiration of mitochondria *in vitro* is governed by ADP concentration according to a simple Michaelis-Menten type relationship and is characterized by constants K_m and V_m. In permeabilized muscle cells the affinity of oxidative phosphorylation for exogenous ADP (added into the medium) is quantitatively characterized by an apparent K_m that reflects an averaged accessibility of ADP to mitochondria. The apparent K_m for exogenous ADP in the control of mitochondrial respiration *in vivo* in skinned cardiac fibers and permeabilized isolated cardiomyocytes has been recorded in many laboratories in the range of 234-400·μM and 200-329 μM, respectively (Andrienko *et al.,* 2003). The apparent K_m for exogenous ADP is ~20·μM in the isolated mitochondria *in vitro* (Chance and Williams, 1955; Saks *et al.,* 1991). High apparent K_m in cardiac cells shows diffusion restrictions for exogenous ADP in cardiac cells.

There is a stoichiometric link between the rate of oxidation of carbon fuels, NADH and $FADH_2$ reduction, flux through the electron transport chain, oxygen consumption, oxidative phosphorylation, ATP hydrolysis, actin-myosin interaction, and external contractile power produced by the heart.

1.4. Energy transport out of mitochondria

Coupled cellular respiration and phosphorylation requires an efficient exchange of metabolites between the cytosol and the mitochondrial matrix. This exchange is a potential regulatory event for the mitochondrial function. The inner mitochondrial membrane contains a number of transport proteins from mitochondrial carrier family, which import multiple substrates into mitochondrial matrix and export metabolites. These carriers are involved in transport of adenine nucleotides, anions, phosphate and substrates for the tricarboxylic acid cycle. Most important mitochondrial carrier is the adenine nucleotide translocase (ANT),

also named ADP-ATP carrier, which facilitates exchange of cytosolic ADP for mitochondrial ATP. Cytosolic ADP and P_i are transported across MOM through porin channels (VDAC). the increase of the cardiac workload, signal transduction occurs between the cytosolic energy utilizing compartments and oxidative phosphorylation sites within the mitochondria. This signal (transfer of ADP) must be fast in order to maintain a steady supply of energy and activation of oxidative phosphorylation. Thus, ANT and VDAC play an important role in regulation and coordination of communication between mitochondria and cytosol.

1.4.1. Adenine nucleotide translocase

Adenine nucleotide translocase (ANT) is a ~30 kDa protein, which is integrated into the MIM and catalyses the exchange of ADP and ATP between cytosol and mitochondria. About 10% of the inner membrane proteins are ANT in mitochondria from tissues with high energy requirements (Kramer and Klingenberg, 1989). The ANT is tightly associated with six molecules of cardiolipin, which are needed for translocation activity but not for binding to the MIM (Hoffmann *et al.,* 1994).

Klingenberg (Klingenberg, 1980) has proposed a `single-binding-center gated pore mechanism` of ANT, by which ANT functional unit has one binding site facing either to matrix or the intermembrane space. Based on analytical centrifugation and neutron diffraction experiments performed in the presence of specific inhibitors, ANT is considered to be organized into homodimers (Hackenberg and Klingenberg, 1980). In contrast, biochemical data support the hypothesis that the functional form of ANT would be a homotetramer, each dimer being involved in the transport of one nucleotide, ATP or ADP (Fiore *et al*., 1998). Formation of homotetramers is consistent with structure analysis (Pebay-Peyroula *et al.,* 2003).

While the main function of ANT is the exchange of cytosolic ADP for ATP produced in mitochondria across inner mitochondrial membrane, it also plays an important role in the formation of a non-specific lethal pore (mitochondrial transition pore, PTP) and in regulating mitochondrial membrane permeability during apoptosis. Formation of this pore is regulated by multiple apoptosis modulators, such as proteins from Bax/Bcl-2 family (Belzacq *et al.,* 2003). Anti-apoptotic protein Bcl-2 stimulates translocator function of ANT and Bax stimulates the pore function of ANT (Belzacq, *et al.,* 2003).

ANT operates as antiporter in exchange of adenine nucleotides in strict stoichiometry 1:1, exchanging one ATP molecule for each ADP molecule in opposite directions. The exchange reaction is electrogenic and driven by the membrane potential. The entrance of ADP and exit of ATP is favored, due to the difference in charges (ATP^{4-}, ADP^{3-}) and the mitochondrial membrane potential. The rate of this electrogenic transport is moderate, 1500-2000 molecules per min, and is compensated by the high density of ANT in inner mitochondrial membrane (Belzacq *et al.,* 2002). The ADP/ATP exchange follows the Michaelis–Menten kinetics, with a K_m of 1-10 μM for ADP (Vignais, 1976; Fiore *et al.,* 1998) and a K_m of 1–150 μM for ATP for rat liver ANT (Fiore *et al.,* 1998). ANT a rate limiting factor for the oxidative phosphorylation under various conditions.

The ANT exists in two conformations which are involved in the translocation mechanism — the cytosolic state and the matrix state of ANT (Gropp *et al.,* 1999). ANT has two transport inhibitors, atractylosides and bongkrekic acids, which are used in the studies of nucleotide transport because they stabilize ANT conformations. Atractylosides block ANT

by binding to the cytosolic state; the bongkrekic acids penetrate membrane and binds to the matrix state of ANT (Gropp *et al.,* 1999). The structure of ANT in complex with an inhibitor carboxyatractyloside has been archived by X-ray crystallography at a resolution of 2.2 Å (Pebay-Peyroula *et al.,* 2003).

There is evidence about the existence of the functional coupling between ANT and mitochondrial creatine kinase (MtCK) – (more details in section 1.5.2). Metabolic channeling by functional coupling between ANT and MtCK drives the balance of the reaction between creatine and ATP far towards the creatine phosphate production (Brdiczka *et al.,* 2006). This coupling plays also important role in prevention of the opening of the PTP, which has a critical role for cell life.

1.4.2. The mitochondrial porin

The MOM is highly permeable for substrates and products up to 5 kDa. The major transport channel in the MOM is mitochondrial porin, also called the mitochondrial voltage-dependent anion-selective channel (VDAC) on the basis of their electrophysiological characteristics (Colombini, 1979). VDAC is a 30–32 kDa polypeptide that contains ~280 amino acids and forms anion-selective channel, which is responsible for most of the metabolite flux across the MOM (Colombini, 1979; Komarov *et al.,* 2005). VDAC is present in high density, 10^3-$10^4/\mu m^2$ (Guo *et al.,* 1995). VDAC protein forms a β-barrel comprised of one α-helix and 16 transmembrane *β*-strand. 3D molecular structure of VDAC is still unknown but the hypothetical 3D model compatible with VDAC sequences and experimental data has been proposed by De Pinto´s group (Casadio *et al.,* 2002).

The VDAC has multiple conformational states with different permeability and selectivity. The transition between the open and closed states is voltage dependent. VDAC has opened (high conductance) state when membrane potential is below 30 mV and closed (low conductance) state, when membrane potential exceeds 30 mV (Komarov *et al.,* 2005). Colombini´s research group has shown with VDAC *in vitro* (Rostovtseva and Colombini, 1996) and in isolated mitochondria (Vander Heiden *et al.,* 2000) that VDAC has weak anionic selectivity in opened state (permeable for ATP and PCr) and cationic selectivity in closed state (permeable for cations, impermeable for anions). Permeability of VDAC for uncharged molecules such as Cr is unaffected by the state of channel. Thus PCr production is not limited by Cr transport across MOM (Vander Heiden *et al.,* 2000). Exact mechanism of VDAC is not clear. The existence of the mobile positively charged voltage sensor is proposed to form part of the channel wall in opened state and to move out of the channel lumen in closed state (Rostovtseva and Colombini, 1996; Komarov *et al.,* 2005).

The existence of a nucleotide-binding site in VDAC is proposed to alter gating of the channel by changing conductance and selectivity because the nucleotides (NADPH, NADH, NAD^+, ATP, ADP, AMP) are able to reduce conductance of channel and change its selectivity (Kromarov *et al.,* 2005). This is supported by the fact that porin channels bind strongly ATP in stochiometry 1:1 (Rostovtseva and Bezrukov, 1998). This binding site is hypothesized to play a crucial role providing sufficient nucleotide translocation capacity under conditions of high energy demand when the rate of flux through VDAC becomes limiting (Gellerich *et al.,* 1993). However, studies with mitochondria, reconstituted systems, and chemically skinned muscle fibers suggest that VDAC permeability is regulated and can constitute a rate-limiting diffusion barrier.

It has been shown *in vitro* that VDAC forms structural complexes with proteins from cytosol and intermembrane space, such as hexokinase, mitochondrial creatine kinase (MtCK), ANT and cytochrome c (Brdiczka *et al.,* 1994*,* 2006). Interactions between VDAC and ANT lead to formation of a dynamic complex that establishes contact sites between the outer and inner mitochondrial membranes. VDAC-ANT complex may exist separately or connected either to the hexokinase or MtCK. The number of contact sites is increased in the presence of ADP (Brdiczka *et al.,* 2006) which allows direct channeling of mitochondrial ATP and cytoplasmic Cr and more efficient synthesis of PCr (Brdiczka *et al.,* 1994; Wallimann *et al.,* 1992). The interaction between VDAC and MtCK induces octamerization of MtCK and affects conductance of porin (Brdiczka *et al.,* 1994).

The VDAC has also been associated with apoptosis in different models. One of the models proposes that VDAC is a component of the PTP, but experimental data are contradictory: some researchers propose structural involvement of VDAC in formation of the permeability transition pore and others propose only modulating role of VDAC in the formation of the PTP (Halestrap, 2002). A second model proposes the involvement of VDAC in the cytochrome *c* release through MOM after activation by Bax (Shimizu *et al.,* 1999). According to a third model the closure of VDAC channel prevents the efficient exchange of ATP and ADP between the cytosol and mitochondrial matrix followed by swelling and rupture of the membranes (Shoshan-Barmatz *et al.,* 2006).

1.5. Creatine kinase

Creatine kinases (CK) are guanidino kinases that catalyze reversible phosphate transfer reaction (Lohmann, 1934), which plays a central role in the intracellular energy transfer:

$$MgADP^{-} + PCr^{2-} + H^{+} \leftrightarrow MgATP^{2-} + Cr$$

CK isoenzymes are separated into two groups, cytosolic CK and mitochondrial CK (MtCK). Three cytosolic and two mitochondrial isoenzymes of CK are known in mammals. CK isoforms are encoded by four independent genes, each coding different monomers for isoenzymes. Cytosolic CK isoenzymes are muscle type (MM-CK), brain type (BB-CK) and hybrid type (MB-CK) (Eppenberger *et al.,* 1967; Yamashita and Yoshioka, 1991). Muscle specific CK isoform (MM-CK) is predominantly found in adult skeletal and cardiac muscles, brain type CK (BB-CK) isoenzyme is highly expressed in brain and neonatal skeletal and cardiac muscles. MtCK isoenzymes are ubiquitous (uMtCK) and sarcomeric (sMtCK) (Wyss *et al.,* 1992). sMtCK expression is restricted to heart and skeletal muscle, but uMtCK is present in many tissues, especially brain and smooth muscle (Wallimann *et al.,* 1992; Schlattner *et al.,* 2006).

All CK isoenzymes have a specific function and subcellular localization in the skeletal and heart muscle cells. MtCK occurs in cristae and peripheral intermembrane space of mitochondria. MM-CK isoforms are structurally coupled to the myofibrils, to the membrane of SR and to the sarcolemma in muscle cells to raise local ATP/ADP ratio and free energy availability. CK isoenzymes are functioning as energy buffering and transport systems between the sites of ATP production and consumption forming microcompartments of ATP (Saks *et al.,* 1994) and are contributing to the regulation of cellular energy homeostasis (Brdiczka *et al.,* 1994).

The activity of MtCK increases and the total activity of CK decrease in the following order: fast-twitch skeletal muscle, slow-twitch skeletal muscle, cardiac muscle. MtCK

activity reaches 30-40 % of total enzyme activity in heart. This specialization comes from metabolic characteristics; fast-twitch muscles rely on glycolytic metabolism and have high activity of MM-CK, but low activity of MB-CK (and phosphocreatine shuttle), while slow muscles have high MB-CK activity (Yamashita and Yoshioka, 1991).

1.5.1. Mitochondrial creatine kinase

MtCK forms a highly symmetrical, cube-like octamers from four dimers (Figure 8C) with a central 20 Å wide channel (Fritz-Wolf *et al.,* 1996). Molecular mass of rat heart MtCK dimer is 82-87 kDa and octameric form 340-345 kDa (Wyss *et al.,* 1992). X-ray structure of octameric MtCK was solved by Fritz-Wolf (Fritz-Wolf *et al.,* 1996). Octameric MtCK has high affinity for cardiolipin and binds tightly to cardiolipin, an acidic phospholipid that is specific for the mitochondrial inner membrane. Therefore, MtCK octamers are bound to the inner mitochondrial membrane in the mitochondrial intermembrane space and in the cristae space (Wallimann *et al.,* 1992; Schlattner *et al.,* 1998, 2004). The height of intermembrane space has been measured to be 9 nm (Fritz-Wolf *et al.,* 1996) and the side length of the cube-like octameric MtCK molecule 9.3 nm (Schlattner *et al.,* 1998), thus the octameric MtCK just fits between the two mitochondrial membranes (Mannella *et al.,* 2001). Soluble MtCK dimers occur in the intermembrane space. CK Octamer/dimer equilibrium *in vitro* is depending on various parameters, such as MtCK concentration, pH, and temperature (Schlattner *et al.,* 1998). Formation of membrane bound octameric state of MtCK *in vivo* is strongly favored by high MtCK concentration, neutral pH, the large membrane surface in the intermembrane space and high affinity to cardiolipin (Schlattner *et al.,* 2004).

MtCK octamer is co-localized with ANT (which is situated in cardiolipin membrane patches (Schlattner *et al.,* 2004)) in the cristae and intermembrane space (Wallimann *et al.,* 1992; Schlattner *et al.,* 1998*,* 2004) and this leads to functional interaction — metabolite channeling between both proteins (Schlattner *et al.,* 2004). The MtCK and ANT are associated in stoichiometry 1:2 (Kuznetsov and Saks, 1986). MtCK interacts with outer membrane phospholipids and the VDAC (Brdiczka *et al.,* 1994; Kottke *et al.,* 1994; Schlattner *et al.,* 1998, 2001, 2004), thus cross-linking the inner and outer membrane and leading to the formation of mitochondrial contact sites (Rojo *et al.,* 1991; Brdiczka *et al.,* 1994; Biermans *et al.,* 1990). Frequency of the formation of the contact sites depends on the metabolic activity of the cell and is induced by the presence of mitochondrial mitochondrial precursor proteins or ADP (Schlattner *et al.,* 2006). Charged N-terminal residues of sMtCK are required for stable octamer formation *in vitro* and may facilitate binding to ANT, cardiolipin, or VDAC to form the contact sites and allowing metabolic channeling *in vivo* (Khuchua *et al.,* 1998).

Formation and regulation of proteolipid complexes are not known, but micromolar concentrations of Ca^{2+} increase the amount of interactions between octameric Mt-CK and VDAC, indicating a physiological regulation of complex formation (Schlattner *et al.,* 2001). The limited permeability of VDAC and the entire outer membrane is taking part in a dynamic microcompartmentation of metabolites in the intermembrane space (Gellerich *et al.,* 1987). These complexes mediate direct channeling of metabolites and microcompartmentation of ATP. This contributes to MtCK-linked channeling and separates mitochondrial ATP and ADP pools (Andrienko *et al.,* 2003). In contact site complexes, this substrate channeling allows a constant supply of substrates and removal of products at the

active sites of MtCK. In cristae complexes, only ATP/ADP exchange is facilitated through direct channeling to the MtCK active site, while Cr and PCr have to diffuse along the cristae space to reach VDAC.

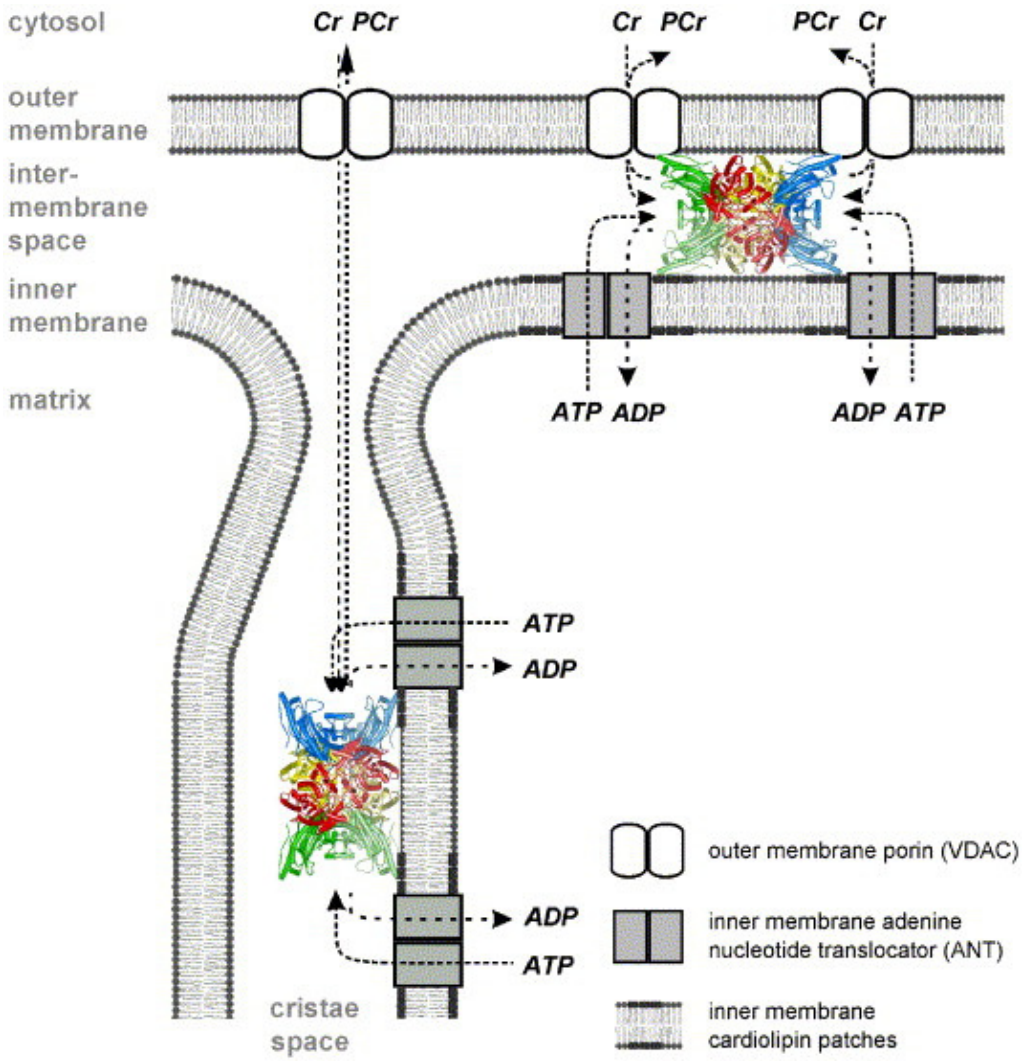


Figure 8 The structure and localization of MtCK and its proteolipid complexes. Dual localization of MtCK and metabolite routes: Octamers of MtCK are bound to mitochondrial membranes by cardiolpin and form proteolipid complexes with VDAC and ANT ("contact site complexes", top right) or with ANT alone ("cristae complexes", bottom center). Interaction of MtCK with ANT is most likely indirect and involves common cardiolipin patches (dark rectangles), while interaction with VDAC is direct and regulated by Ca^{2+}. The proteolipid complexes allow a direct exchange of MtCK substrates and products (substrate or metabolite channeling, depicted by arrows). In contact site complexes (top right), this substrate channeling allows for a constant supply of substrates and removal of products at the active sites of MtCK. In cristae complexes (bottom center), only ATP/ADP exchange is facilitated through direct channeling to the MtCK active site, while creatine (Cr) and phosphocreatine (PCr) have to diffuse along the cristae space to reach VDAC (from: Schlattner *et al.,* 2004, 2006 with permission)

1.5.2. Functional coupling between ANT and MtCK

The coupled ANT-MtCK system is an excellent example of the general phenomenon of functional coupling in supramolecular complexes of enzymes, transporters, which can be described by the following formula (Saks *et al.,* 2004):

Functional coupling = Metabolic channeling + Microcompartmentation

First indications of functional coupling between MtCK and mitochondria were published in 1939 when Belitzer and Tsybakova showed that in muscle homogenates the oxygen consumption was stimulated by creatine and always resulted in phosphocreatine (PCr) production with the ratio of PCr/O about 3 (Belitzer and Tsybakova, 1939). Most of information about the functional role of the mitochondrial creatine kinase has been obtained in studies of heart mitochondria, sMtCK and to a lower extent in skeletal muscle sMtCK, and also in brain and smooth muscle mitochondria, uMtCK, sMtCK and in some lesser extent in skeletal muscle sMtCK and also for brain and smooth muscle mitochondria, in both cases uMtCK (Schlegel *et al.,* 1988; Haas and Strauss, 1990; Qin *et al.,* 1998; Schlattner *et al.,* 2000). The work of Belitser and Tsybakova on muscle homogenates showing constant PCr/O_2 ratio was first to describe the activation of respiration by creatine, due to creatine kinase reaction, as it was already mentioned above (Belitzer and Tsybakova, 1939). Bessman and Fonyo showed in isolated heart muscle mitochondria that addition of creatine increased the respiration rate in the State 4 (presence of ATP) (Bessman and Fonyo, 1966). Similar data were reported by Vial *et al.* (Vial *et al.,* 1972). In 1973, Jacobus and Lehninger studied the kinetics of the stimulatory effect of creatine on the State 4 respiration rate and found that at its physiological concentration, 10-15 mM, creatine stimulated the respiration maximally, to the State 3 level (Jacobus and Lehninger, 1973). From this important work, the ideas of coupling of the mitochondrial creatine kinase reaction with the oxidative phosphorylation as a mechanism of regulation of respiration started to take a shape. In 1974 Saks *et al.* published a paper (Saks *et al.,* 1974) confirming the results reported by Jacobus and Lehninger, and in 1975 the same authors applied the kinetic analysis and simple methods of mathematical modeling to investigate the phosphocreatine production coupled to the oxidative phosphorylation (Saks *et al.,* 1975). The results showed that the oxidative phosphorylation itself controls the phosphocreatine production in heart mitochondria. When uncoupled from oxidative phosphorylation (if the latter is not activated, for example), the mitochondrial creatine kinase reaction does not differ kinetically and thermodynamically from other creatine kinase isoenzymes: the reaction always favours the ATP production and according to the Haldane relationship, ADP and phosphocreatine binding is more effective due to higher affinities than that of ATP or creatine, respectively (Saks *et al.,* 1974, 1975). When the calculated predicted rates of the reaction were compared with the experimental ones, good fitting for any experimental conditions was found in the absence of oxidative phosphorylation but not when the latter was activated: under conditions of oxidative phosphorylation the mitochondrial creatine kinase reaction was strongly shifted in direction of phosphocreatine synthesis (Saks *et al.,* 1975).This was taken to show that ATP produced in mitochondrial oxidative phosphorylation was much more effective substrate for MtCK than the MgATP in medium, and it was proposed that this is due to direct transfer of ATP by adenine nucleotide translocase from matrix space to the creatine kinase, which should be

located somewhere in the close proximity to ANT to make this direct channeling possible (Saks *et al.,* 1975). To understand better the mechanism of this phenomenon, Jacobus and Saks undertook a joint study and performed a complete kinetic analysis of the creatine kinase reaction in isolated rat heart mitochondria under both conditions: with and without oxidative phosphorylation (Jacobus and Saks, 1982). While the kinetic constants for guanidino substrates - creatine and phosphocreatine - were not changed and were the same in both conditions, the oxidative phosphorylation had a specific effect on the kinetic parameters for adenine nucleotides. Under conditions of oxidative phosphorylation the dissociation constants can be measured only for a substrate - MgATP - in the medium, and the apparent affinity for this substrate (if creatine was already bound to MtCK) was seen to be increased by an order of magnitude (Jacobus and Saks, 1982). The Haldane relationship for the creatine kinase reaction was no more valid, showing the involvement of some other processes – oxidative phosphorylation and ANT (Jacobus and Saks, 1982). The explanation proposed was the direct transfer of ATP from ANT to MtCK due to their spatial proximity which results also in increased uptake of ADP from MtCK (reversed direct transfer), and as a result, the turnover of adenine nucleotides is increased manifold at low external concentration of MgATP, this maintaining high rates of oxidative phosphorylation and coupled phosphocreatine production in the presence of enough creatine. This was the intuitive hypothesis of the direct transfer of ATP and ADP as a coupling mechanism for qualitative explanation of the decrease of the apparent (under these conditions) kinetic constants for MgATP in the MtCK reaction in the presence of oxidative phosphorylation (Saks *et al.,* 1974*,* 1975, Jacobus and Saks, 1982). Further experiments confirmed these conclusions and in recent structural studies and in mathematical modeling of functional coupling, interesting quantitative features of this mechanism were revealed.

The conclusions of the privileged access of mitochondrial ATP to MtCK and increased mitochondrial turnover of adenine nucleotides in the presence of creatine were directly confirmed by Barbour *et al.* with the use of isotopic method (Barbour *et al.,* 1984) and by the thermodynamic approach by De Furia (De Furia *et al.,* 1980), Saks *et al.* (Saks *et al.,* 1985), and Soboll *et al.* (Soboll *et al.,* 1994). Finally, an effective competitive enzyme method for studying the functional coupling phenomenon, namely the pathway of ADP movement from MtCK back to mitochondria, was developed by Gellerich *et al.* (Gellerich and Saks, 1982; Gellerich *et al.,* 1987; 1994, 1998, 2002). These authors used the phosphoenol pyruvate (PEP) – pyruvate kinase (PK) to trap ADP and thus to compete with ANT for this substrate. This competitive enzyme system was never able to suppress more than 50 % of the creatine - stimulated respiration in isolated heart mitochondria, this showing the rather effective channeling of ADP from MtCK to the ANT (Gellerich and Saks, 1982). The Gellerich group has preferred to explain these latter data by the hypothesis of dynamic compartmentation of adenine nucleotides in the intermembrane space, that meaning that there is some control of the permeability of the MOM and because of this, the formation of some ADP and ATP concentration gradients (Gellerich *et al.,* 1987; 1994, 1998, 2002). This was an alternative hypothetical mechanism of coupling between MtCK and ANT without direct transfer of the substrates. Interestingly, this hypothesis focused attention on the role of MOM in the control of mitochondrial function, and foresaw many important aspects of the control of mitochondrial function in vivo, but appeared to be insufficient to explain quantitatively the functional coupling between MtCK and ANT.

Tight functional coupling of ANT and MtCK is important for regulation of metabolism and it is the basis of metabolic stability of (Saks *et al.,* 1975, 1985, 1994; Jacobus and Saks, 1982; Spindler *et al.,* 2002) of the cells with high energy demand, such as heart and brain. Functionally coupled MtCK and ANT, are able to delay or even prevent Ca^{2+}-induced opening of the mitochondrial PTP. Decrease in the functional coupling between MtCK and ANT (decreased PCr/ATP ratio), and also increased permeability of the MOM to ADP are first detectable signs during ischemia (Spindler *et al.,* 2002). Recent results with brain and liver mitochondria show importance of functional coupling (MtCK activity) in reduction of reactive oxygen species (ROS) generation in mitochondria and this antioxidant role depends on the phosphocreatine (PCr)/Cr ratio (Meyer *et al.,* 2006).

1.6. Energy transfer networks and channeling of energetic substrates in cardiac cells

The need for energy in cardiac muscle is dependant on cardiac workload, which may very ~20 fold. From the point of view of regulation of biochemical reactions the most noteworthy aspect of energy production in the cardiac muscle is that the respiration rate in heart cells is increased manifold without any significant change in the intracellular levels of phosphocreatine (PCr) and ATP, which is termed as metabolic stability (Neely *et al.,* 1972; Balaban *et al.,* 1986) or `metabolic homeostasis´ (Balaban *et al.,* 2002). This metabolic stability is achieved with energy transfer networks to overcome intracellular diffusion restrictions due to the macromolecular crowding and intracellular structure.

Mitochondrial ATP is transported to the energy utilization sites in muscle cells mostly via spatially arranged and functionally coupled intracellular enzymatic networks (also called phosphotransfer networks) consisting of creatine kinase, adenylate kinase, carbonic anhydrase and glycolytic enzymes (Figure 9). These networks contribute efficient high-energy phosphoryl transfer and signal communication between ATP-generating and ATP-consuming/ATP-sensing processes to maintain balance between cellular ATP production and consumption (Jacobus, 1985; Wallimann *et al.,* 1992; Saks *et al.,* 1994, 2006; Joubert *et al.,* 2002; Dzeja and Terzic, 2003). Phosphotransfer networks are needed to overcome diffusion restrictions in cells.

There is increasing evidence for a restriction of ATP (and ADP) diffusion in the cell, particularly pronounced in the subsarcolemmal area (Vendelin *et al.,* 2004a). These restrictions are bypassed by the CK–PCr system due to the attachment of the MM-CK isoenzyme to the sarcolemma. It was shown directly that the CK system maintains a close functional coupling between mitochondria and ion channels in sarcolemma.

The major energy transfer systems in cells with high energy demand, like muscle and brain, are creatine kinase and adenylate kinase systems. The CK system relies on specifically localized isoenzymes of CK and the rapidly diffusible phosphocreatine (PCr) as a 'high-energy' intermediate. The existence of direct transfer of PCr by phosphocreatine shuttle helps to: (i) overcome intracellular diffusion restrictions in cytosol for ATP (diffusion constants for PCr and Cr are higher compared to adenine nucleotides); (ii) avoids inhibition of ATPases by accumulation of reaction product, ADP and (iii) maintain the metabolic homeostasis in cardiac muscle cells without any change in the concentration of the substrate pool in macrocompartments.

The CK system is composed of several isoenzymes of CK with specific localization at distinct sites and interaction with a number of cellular proteins, enzymes, biological

membranes or organelles (Figure 9) and are forming PCr channeling circuit (PCr shuttle), which is connecting ATP production and utilization sites. A significant amount of the nonmitochondrial CK (MM-CK) is structurally and functionally: (1) associated with the sarcomeric M-band of the myofibrils by four MM-CK-specific lysine residues (Hornemann *et al.,* 2003; Schlattner *et al.,* 2004) for local regeneration of ATP for myosin-ATPases during the contraction cycle (Wyss *et al.,* 1992; Ventura-Clapier, *et al.,* 1994); (2) co-localized with different ion pump-ATPases (K^+/Na^+-ATPase, Ca^{2+}-ATPase) in the plasma membrane (Brdiczka *et al.,* 1994); (3) co-localized with the , Ca^{2+}-pump of the SR (SR Ca^{2+}-ATPase pump) (Rossi *et al.,* 1990; Wallimann *et al.,* 1992). Tight functional coupling of CK to ATPases provides the maximal free energy of ATP and efficiency of myosin ATPase (also ion ATPase-pumps) by producing locally very high ATP/ADP ratio and avoids accumulation of reaction products ADP and H^+ and thus inhibition of ATPases (Wallimann *et al.,* 1992; Wyss and Kaddurah-Daouk, 2000; Dzeja and Terzic, 2003).

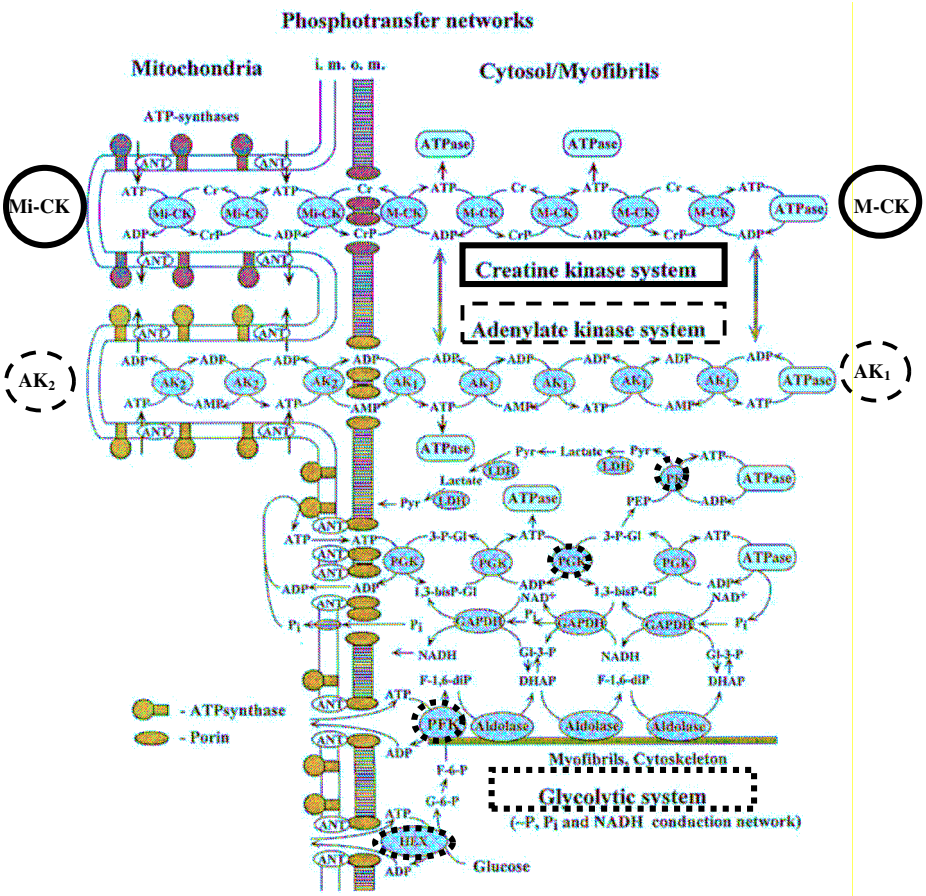


Figure 9 Phosphotransfer networks. Main ATP-delivering network in cardiac cells is consisting of isoenzymes of creatine kinase (CK), which are connecting mitochondria (Mi-CK) and ATP–consuming processes in cytosol, myofibrils (M-CK) to maintain global cytosolic ATP/ADP ratios. Adenylate kinase system is alternative (from Dzeja *et al*., 2004 with permission)

PCr channeling circuit represents an efficient regulator of energy flux and uses metabolite channeling as a fine-tuning device for local ATP levels. Functioning of PCr shuttle is based on its high sensitivity towards ADP and it prevents accumulation of ADP (and P_i) and avoids inhibition of ATPases in excitable cells (Wallimann *et al.,* 1992; Dzeja *et al.,* 1998; Dzeja and Terzic, 2005). The CK reaction maintains a low [ADP] and [Pi], thereby maximizing the phosphorylation potential (Saupe *et al.,* 2000).

Understanding of creatine kinase function was limited, when the cell was considered as a homogenous system (well mixed bag) where enzymes are in equilibrium, and metabolites have uniform distributions and concentrations (Meyer *et al.,* 1984; Kushmerick, 1995).

$$[ADP] = [ATP][Cr]/[PCr]K'_{eq}$$

This contradicts to experimental observations, since intracellular ADP concentration calculated from this equilibrium equation does not change under conditions of metabolic stability (constant PCr, Cr and ATP concentrations) when the oxygen consumption rate increases 20 times (see below, next section). The reaction has an equilibrium constant of 160 in the direction of ATP formation (Teague and Dobson, 1992; Golding *et al.,* 1995).

Recently, a new experimental approach that allows quantification of unidirectional fluxes of creatine kinase localized in different subcellular compartments provided strong evidence for the involvement of creatine kinase in intracellular energy transfer (Joubert *et al.,* 2002). Studies with transgenic animals demonstrate that creatine kinase deficiency compromises energy delivery for muscle contraction, intracellular calcium handling and signal communication to membrane metabolic sensors, such as the K_{ATP} channel (Steeghs *et al.,* 1997; Kaasik *et al.,* 2001; Abraham *et al.,* 2002). In creatine kinase-deficient muscles, phosphotransfer catalyzed by adenylate kinase as well as by glycolytic enzymes provide the major route for intracellular high energy phosphoryl transfer (Dzeja *et al.,* 1998, 2003; de Groof *et al.,* 2001). Such alternative high-energy phosphoryl routes may rescue cellular bioenergetics in cells with compromised creatine kinase (CK)-catalyzed phosphotransfer (Dzeja *et al.,* 2000).

Adenylate kinase-catalyzed reversible phosphotransfer between ADP, ATP and AMP molecules has been implicated in processing metabolic signals associated with cellular energy utilization (Noda, 1973; Dzeja *et al.,* 1998). The enzyme adenylate kinase also functions to maintain high levels of ATP by transferring phosphoryl groups among the adenine nucleotides: $2ADP \Leftrightarrow ATP + AMP$

Adenylate kinase system is regulated through high sensitivity towards AMP (Dzeja *et al.,* 1998; Dzeja and Terzic, 2005). In case of AMP accumulation AMP-activated protein kinase (AMPK) and other AMP-sensitive components would be activated by free AMP initiating signaling cascades that would turn on compensatory mechanisms for increasing energy supply and reducing energy consumption (Pucar *et al.,* 2004; Kahn *et al.,* 2005).

Facilitated communication between cellular energy transforming and consuming processes minimizes metabolite gradients, reducing energy dissipation and providing a capability to direct high-energy phosphoryl groups into specific pathways according to physiological needs. Derangement in cellular energy flow and distribution has been implicated in cardiovascular (Dzeja *et al.,* 2000; Perez-Terzic *et al.,* 2001) and neuro-degenerative diseases (Ames, 2000). These findings emphasize the importance of creatine kinase in providing energetic efficiency in support of various cellular functions.

1.7. Cardiac energetics: Frank-Starling mechanism in heart

The ability of the heart to change its force of contraction and therefore stroke volume in response to changes in venous return is called Frank-Starling mechanism (or Starling's Law of the heart), in honor to Otto Frank and Ernest Starling. Otto Frank found using isolated frog hearts that the strength of ventricular contraction was increased when the ventricle was stretched prior to contraction. This observation was extended by the studies of Ernest Starling and colleagues (in the early 20th century), who used heart–lung preparations and measured the rate of oxygen consumption as a measure of 'the total energy set free in the heart during its activity' and discovered, that both work and respiration rates, increased linearly with increase of left ventricular end-diastolic volume (Starling and Visscher, 1926).

The rate of oxygen consumption (VO2) is main parameters of heart energetics and is described as a linear function of the pressure–volume area (PVA):

$$V\mathrm{O}_2 = \mathrm{A} \times \mathrm{PVA} + D,$$

where, D is unloaded $V\mathrm{O}_2$, PVA consists of the mechanical work within the contraction cycle, and A is a constant (which inversely describes the efficiency of the heart) (Suga, 1990). It was discovered later that linear relationship between oxygen consumption and workload of the heart occurs under conditions of metabolic stability of the heart — in the absence of measurable changes in the ATP and PCr cellular content (Neely *et al.,* 1972).

Williamson and colleagues have shown that the rate of respiration in isolated working hearts can be changed by more than an order of magnitude, ~15–20 times, from an unloaded $V\mathrm{O}_2$ of around 8–12 μmol min^{-1} (g dry wt)$^{-1}$ to a maximal value of 170 μmol min^{-1} (g dry wt)$^{-1}$, under conditions of metabolic stability (Williamson *et al.,* 1976). This observed metabolic stability or homeostasis is underlying Frank-Starling law and is also referred as the 'stability paradox' (Hochachka, 2003).

Frank-Starling mechanism is a fundamental principle in cardiac physiology, the basic adaptation of the heart to an increased ventricular filling (end-diastolic volume) and therefore preload - adaptive response to an increase in demand (Katz, 2002). In cellular level this increased volume is stretching myocyte and increases sarcomere length (SL), which causes an increase in force generation. The latter enables the heart to eject the additional venous return and increase stroke volume. This phenomenon is described by the length-tension and force-velocity relationships for cardiac muscle. Increasing preload increases the active tension developed by the muscle fiber and increases the velocity of fiber shortening at a given afterload. The effect of increased sarcomere length on the contractile apparatus is termed length-dependent activation. Despite the numerous studies, the molecular mechanism of length-dependent activation is still not fully understood.

The relationship between SL and the maximal force was described by Gordon *et al.* in the cross-bridge model of skeletal muscle contraction: the maximal force at any SL is determined by degree of overlap of the thick and thin filaments (i.e. how many crossbirdges can cycle) (Gordon *et al.,* 1966). Cardiac muscle has strong parallel elastic component which prevents sarcomere lengths >2.3 μm (so called `descending limb´), which could result in progressive decrease in contraction and failure in cardiac output. Length-tension relationship is steeper for cardiac cells. Heart functions as `ascending limb´ of the length-tension relationship to ensure increased contraction with increase in SL — thick and thin filaments are in full-overlap state or close to it at SL of ~1.8-~2.3 μm (Gordon *et al.,* 1966) and twitch force increases several fold when stretched within the working SL range observed in heart.

Changes in myofilament overlap may only explain ~20% of the classic Frank-Starling effect (Bers, 2001).

Hibberd and Jewell showed that with an increase in sarcomere length from 1.9 to 2.4 μm there was an increase in Ca^{2+} sensitivity (Hibberd and Jewell, 1982). Several studies later have confirmed that a length-dependent shift in Ca^{2+} sensitivity is the key component of the Frank-Starling relation. Increased SL increases troponin C Ca^{2+} sensitivity, which increases the rate of cross-bridge attachment and detachment, and the amount of tension developed by the muscle fiber. The effect of Ca^{2+} sensitivity is greater in cardiac muscle and contributes to its greater length dependency of activation.

Fukuda *et al.* (Fukuda *et al.,* 2000) reported that length-dependent activation can be modulated at the level of cross-bridges, which interact with actin at longer SL due to length-dependent reductions in lateral spacing between thick and thin filaments due to closer proximity to actin more crossbridges bind and thereby increase contractile force (Fuchs and Smith, 2001). The probability of crossbridge formation is likely to increase when the distance between myosin and actin decreases via reduction of interfilament lattice spacing (i.e., radial separation between thick and thin filaments) due to closer proximity to actin more crossbridges bind and thereby increase contractile force (Fuchs and Smith, 2001; Fukuda and Granizier, 2005). The reduction in Ca^{2+} sensitivity with decreasing sarcomere length may be explained by increased distance between thick and thin filament (lattice spacing). Strongly attached cross-bridges contribute to activation, along with Ca^{2+} binding, and the probability of these attachments at a given $[Ca^{2+}]$ decreases with increasing lattice spacing.

Konhilas *et al.* (Konhilas *et al.,* 2002) have shown that the myofilament lattice spacing and Ca^{2+} sensitivity are not well correlated and alterations in myofilament lattice spacing may not be the primary the mechanism that underlies the SL-induced alteration of Ca^{2+} sensitivity in myocardium.

Another hypothesis is that the increase in Ca^{2+} sensitivity at stretched lengths may involve positive cooperativity in crossbridge binding to actin (Robinson *et al.,* 2002), ie, initial crossbridge binding facilitates further binding that in turn increases force at any given $[Ca^{2+}]$ (Fitzsimons and Moss, 1998). Consistent with this idea, bathing skinned myocardium with a strong-binding derivative of myosin substantially reduces the length-dependent changes in Ca^{2+} sensitivity of force. Presumably, application of the strong binding derivative more nearly saturates the cooperativity of crossbridge binding, so that the activation of force does not vary as much with muscle length. The mechanism of increased cooperativity at long lengths might be due to increased probability of initial crossbridge binding to actin due to reduced lattice spacing or an effect of stretch on crossbridge disposition (Moss and Fitzsimons, 2002). This is supported by recent evidence, which indicate that titin/connectin plays an important role in length-dependent activation by sensing stretch and promoting actomyosin interaction (Fukuda and Granizier, 2005). Titin/connectin is involved in active force generation by modulation of interfilament lattice (Fukuda *et al*., 2001; Fukuda and Granizier, 2005). Although, this giant elastic protein, titin/connectin, was usually regarded as a passive force generator in the sarcomere that is not involved in active force generation in vertebrate striated muscle, but biochemical analyses have revealed that titin/connectin interacts with myosin and myosin-binding C *in vitro* (Freiburg and Gautel, 1996). It has also been shown that titin/connectin binds to actin in and near the Z-line (Trombitas and Granzier, 1997). Titin/connectin produces radial as well as longitudinal force, in the cardiac myofilament lattice and the former may pull the thin filament closer to the thick filament,

resulting in a reduction of the lattice spacing and, hence, an increased likelihood of actomyosin interaction.

1.8. Regulation of respiration *in situ*

Major amount of ATP in cardiac cells is produced in mitochondria by oxidative phosphorylation and ATP is consumed for contraction by actomyosin ATPases and for ion transport by ATPase pumps. The need for ATP in cardiac cells is not constant; it is depending on activation of the contractile apparatus and cardiac work output, which may change manifold. But intracellular [ATP] and [PCr] are constant (10 mM and 30-35 mM, respectively (Wyss *et al.,* 1992)), even when respiration rate in cardiac muscle is increased manifold. The energetic state of the heart is not defined simply by the concentration of ATP. In fact, the amount of ATP made and used per minute (turnover) is many times greater than the size of the ATP pool. Constant and high ATP supply is critically important for maintaining cardiac performance. This metabolic stability (Neely *et al.,* 1967, 1972; Balaban *et al.,* 1986) in cardiac cells is ensured by highly regulated and integrated energy transfer networks, which are orchestrating communication between ATP-producing and -consuming compartments and thus, regulating metabolic state of cardiac cells. Therefore, very important question in bioenergetics is: how oxidative phosphorylation (mitochondrial respiration) is regulated *in vivo/in situ*?

1.8.1. The mitochondrial calcium cycle and respiration regulation paradox

Currently accepted mechanism in regulation of mitochondrial respiration is the assumtion of parallel activation of contraction and respiration by calcium (Territo *et al.,* 2001; Balaban, 2002). This theory proposes that cytosolic Ca^{2+} regulates both the utilization of ATP by the work at the myofilaments and ion transport by the SR and sarcolemmal membrane pumps are thought to be stimulated by Ca^{2+} in parallel with mitochondrial ATP production (Balaban, 2002).

This theory is supported by the fact that contraction is activated by Ca^{2+}-induced Ca^{2+} release from SR and binding to troponin C in the troponin–tropomyosin complex of thin filaments (Gordon *et al.,* 2001) and it assumes that Ca signals match with the demand for ATP in the cell to its production in mitochondria, and thus to the control of respiration (Territo *et al.,* 2001; Balaban, 2002; Gunter *et al.,* 2004). From the other side the activation of ATP production in mitochondria is supported by the fact that Ca^{2+} is an activator of the TCA cycle dehydrogenases and is thus increasing the capacity of oxidative phosphorylation (McCormack *et al.,* 1990; Hansford and Zorov, 1998; Balaban, 2002) and Ca^{2+} is suggested to modulate F_oF_1-ATPase activity (Das and Harris, 1990; Harris and Das, 1991; Territo *et al.,* 2000). Concentration of intracellular Ca^{2+} transients should change 15 to 20 fold, but there is evidence that Ca^{2+} transients do not change during alteration of heart workload, and the only activator role of Ca^{2+} on regulation of respiration in mitochondria is under doubt (Shimizu *et al.,* 2002).

Quantitative estimations of calcium effects on mitochondria are in conflict with the magnitude of changes in the respiratory rate *in vivo*. Both, experimental studies of Ca^{2+} effects on the mitochondrial respiration *in vitro* (Territo *et al.,* 2001) and mathematical modeling of mitochondrial metabolism (Cortassa *et al.,* 2003) have shown that changes in Ca^{2+} concentration can at maximum double respiration. Thus, this degree of activation of

mitochondrial respiration is not sufficient to explain the energy flux changes of more than an order of magnitude that are observed in muscle cells *in vivo.*

In cells with small fluctuations of energy fluxes, direct regulation of mitochondrial activity by Ca^{2+} may be sufficient (Jouaville *et al.,* 1999), but for excitable cells with high and rapidly fluctuating energy fluxes, such as the heart, skeletal muscle, brain and other cells, this is not the case.

1.8.2. Respiration regulation in situ

An apparent K_m for exogenous ADP in regulation of mitochondrial respiration in cardiac cells is high, ~250-400 μM (Andrienko *et al.,* 2003) — an order of magnitude higher than that in isolated mitochondria, ~20 μM (Chance and Williams, 1955; Saks *et al.,* 1991). Thus, *in vitro* methods do not show actual situation *in vivo*, because isolation of mitochondria by homogenization of tissue and sedimentation of mitochondria by centrifugation affect seriously structural and functional properties of the organelles. Disruption of the cellular architecture is likely to alter the parameters concerning the interaction between the mitochondria and the extra mitochondrial space and to destroy the organized structure of cytoplasm. The latter has been simulated for isolated mitochondria with medium containing macromolecules (15 and 20% of dextran) to have oncotic pressure and concentration of macromolecules similar to cytoplasm *in vivo* (Gellerich *et al.,* 1998, 2002). The permeability of MOM for adenine nucleotides decreased and increased the diffusion barrier for adenine nucleotides and the apparent K_m increased from 20 μM to 50 and 122 μM in medium containing 15 and 20% of dextran, respectively (Gellerich, 1998, 2002). This shows existence of severe diffusion restrictions in cytosol of cardiac cells and possible role of cellular structure on regulation of mitochondrial respiration in cardiac cells. This problem is not yet sufficiently explained and has been therefore widely studied during the last decades.

Diffusion restriction comes from the level of the MOM, VDAC. The latter is believed to be controlled by some unknown intracellular factor which may be connected with cytoskeleton and is lost during isolation of mitochondria (Saks *et al*., 1993). This hypothesis is supported by the changing role of cardiac cytoskeleton from purely structural one to the one including regulation of cell function (Katz, 2000).

After ruling out the possible role of myosin, selective barrier function of MOM was taken in to focus. Well-controlled conditions for swelling of heart mitochondria in skinned fibers in hypo-osmotic medium (40 mosM) were used to study possible role of MOM in ADP retarded diffusion. Selective disruption of MOM by hypo-osmotic shock strongly decreased the apparent K_m value in cardiac fibers to ~30 μM, close to the value in isolated mitochondria *in vitro* (Saks *et al.,* 1993*,* 1995). Swelling of MOM and decrease in apparent K_m for ADP is also observed in ischemia (Kay *et al.,* 1997b). This showed that the integrity of the MOM is involved in the phenomenon of high apparent K_m for exogenous ADP and MOM is a major structure which retards the ADP diffusion into the intermembrane space (Saks *et al.,* 1993). Porin pores in the MOM were hypothesized to limit the permeability of ADP in cells *in vivo* (Saks *et al.,* 1993; 1994; 1995).

The role of myosin in regulation of mitochondrial respiration regulation by forming diffusion barrier for exogenous ADP (Saks *et al.,* 1991) has been ruled out after the results in cardiac fibers without myosin (‘ghost fibers’) were obtained (Saks *et al.,* 1993). High apparent K_m for exogenous ADP (~350 μM) has been observed in ghost fibers, despite the

extraction about 70% of myosin filaments from sarcomeres with 0.8 M KCl. The results with 'ghost' cardiac cells were the first basis for conclusion of the possible role of the MOM in limitation of affinity of mitochondria for exogenous ADP *in vivo* (Saks *et al.,* 1993, 1994, 1995). At same time the cell shape, regular arrangement of mitochondria, structures of Z-line and thin filaments were well preserved due to the cytoskeleton system (Saks *et al.,* 1993; Kay *et al.,* 1997a; Appaix *et al.,* 2003).

Short selective proteolytic treatment of skinned cardiac fibers decreased apparent K_m value to 40–98 μM, without significant alteration of V_{max} or the intactness of MOM, as assessed by the cytochrome c test (Kuznetsov *et al.,* 1996; Kay *et al.,* 1997a). Proteolytic treatment with 0.1 μM trypsin for 5 min at 4°C begins to disorganize the intracellular structure and complete disorganization of intracellular structure is achieved with 5 μM trypsin (Saks *et al.,* 2001; Seppet *et al.,* 2001, 2004; Appaix, 2003). These results show that ADP diffusion in cardiac cells *in vivo* may be retarded due to some unknown cytoplasmic trypsin-sensitive protein factor(s) which may be lost during isolation of mitochondria (Kuznetsov *et al.,* 1996; Kay *et al.,* 1997a; Saks *et al.,* 2001). These proteins are considered to be important in the control of the permeability of the MOM *in situ* (Saks *et al.,* 1995) by possible connections between the outer mitochondrial porin pores (VDAC) and a protein associated with cytoskeleton (Kay *et al.,* 1997a). These unknown protein factor(s) may take part in determining localization of mitochondria in the cells by building up structurally and functionally organized pathways of feedback signaling between ATP-consuming (ADP-supplying) systems and mitochondria. Due to such a structural organization, the mitochondria become less easily accessible to exogenous ADP and endogenously generated ADP does not equilibrate easily with the ADP in the medium (Saks *et al.,* 2001).

The role of components of cytoskeleton such as tubulin (main component of microtubular network), desmin (main component of intermediate filaments in cytoskeleton of cardiac muscle, localized in the Z-lines) and plectin (co-localized with desmin), have been under investigation as possibly responsible for control of the permeability of the MOM *in situ*. It has been observed by confocal immunofluorescence imaging that microtubular and plectin networks disappear during short proteolytic treatment and desmin is not significantly affected by trypsin. Possible role of microtubular network in regulation of mitochondrial respiration has been shown as the decrease of apparent K_m correlates with disappearance of tubulin labeling (Appaix, 2003). Desmin has shown to be important in positioning of mitochondria, as its absence results in disorganized structure of cardiac cells with altered mitochondrial population, probably lacking some unknown VDAC controlling protein. Thus, there may be functional connection between mitochondria, cellular structural organization and cytoskeleton in the cells *in vivo* due to the existence of still unidentified protein factor(s) (Kay *et al.,* 1997a).

The existence of ICEU has been proposed to explain diffusion restrictions and substrate channeling in cardiac cells. According to this hypothesis, cardiac cell is divided into autonomous energetic units, which are separated from each other with cytoskeleton forming high compartmentation within cardiac cell with high diffusion restrictions for exogenous adenine nucleotides. Mitochondria are incorporated into functional complexes with sarcomeres and sarcoplasmic reticulum by connection with cytoskeleton. These connections between cytoskeleton and mitochondrial membranes are hypothesized to be important for localization of mitochondria in the cells and may control the permeability of MOM for

exogenous ADP (Saks *et al.,* 2001). These interactions between mitochondria, myofibrils and sarcoplasmic reticulum has been main research object in numerous works lately (Saks *et al.,* 2001; Kaasik *et al*, 2001; Nozaki *et al.,* 2001; Seppet *et al.,* 2001). Transport of energy substrates within these units is organized by direct substrate channeling (functional coupling of enzymes). Metabolites are transported mostly via phosphotransfer networks to overcome diffusion restrictions for ADP and ATP. Phosphotransfer networks are consisting of several creatine kinase isoforms with specific location in cells to connect ATP producing and consuming sites with direct channeling of adenine nucleotides and to regulate metabolic state of cardiac cells by feedback regulation (Saks *et al.,* 2001).

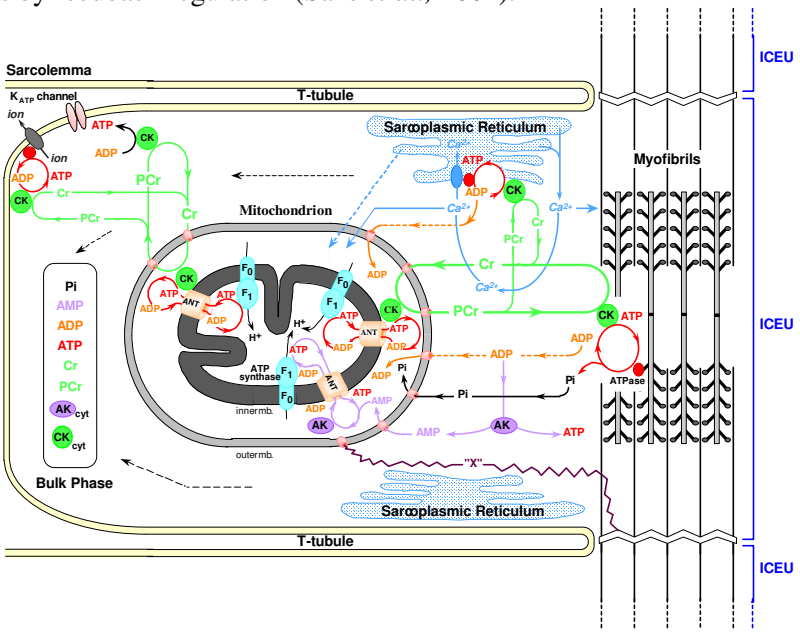


Figure 10 The intracellular energetic units (ICEU) in cardiac cells (Saks, 2001)

1.9. Energetics in brain cells

Energy metabolism of brain has been extensively studied for a long period of time and the results were described in several review articles by Erecinska et al. (Erecinska et al. 1996, 2004), Ames (Ames, 2000), Nicholls (Nicholls, 2003), and the latest "state of art" in the field is available in the new, third edition of the Handbook of Neurochemistry and Molecular Neurobiology (Gibson and Dienel, 2007). In particular, the energy metabolism of the nerve endings – synaptosomes – was studied in much detail by Erecinska and Nicholl's groups (Erecinska et al. 1996; Nicholls, 2003, Scott and Nicholls, 1980). In these studies, the presence of PCr and the CK system in synaptosomes was always noticed, but their roles were not investigated in further detail (Erecinska et al. 1996). CK system was studied in synaptosomes in this work

AIM OF THESIS

The aim of this study was to investigate the role of cellular structure and internal complex cellular organization in regulation of energy metabolism in cells with high metabolic rate, such as cardiac and brain cells. Therefore, cellular structure, respiration regulation, energy transfer by phosphotransfer networks and the role of MtCK in energy metabolism were under investigation.

The following problems were investigated in this work:

1) Comparison of respiration regulation and channeling of endogenous ADP in permeabilized cardiac cells with different cellular organization: isolated adult cardiomyocytes were compared with immortalized cardiac cell line (HL-1 cells) with beating and non beating properties.
2) Role of cellular organization in regulation of mitochondrial respiration in cardiac cells was studied by altering the cellular structure of cardiomyocytes in the result of selective protease digestion
3) The influence of different free Ca^{2+} concentrations on alterations of the structure of cardiac cells, regulation of mitochondrial respiration *in situ* and channeling of endogenous ADP in cardiac skinned fibers, and in their 'ghost' preparations (after selective extraction of myosin) was investigated.
4) Functional coupling between MtCK and ANT (direct transfer of mitochondrial ATP from ANT to the MtCK) was demonstrated. The dissociation constants for both MtCK reaction substrates, ATP and Cr, in isolated heart and brain mitochondria were determined in the presence and absence of oxidative phosphorylation.
5) The role of MtCK in regulation of mitochondrial respiration *in situ* in cardiomyocytes, synaptosomes and NB HL-1 cells was studied.

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (250–350 g) were used throughout the study. Animals were housed five per cage at constant temperature (22±1°C) in environmental facilities with a 12:12 h light-dark cycles schedule and were given standard laboratory chow and tap water *ad libitum*. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85–23, revised in 1985). Animals were anaesthetized with sodium pentobarbital (50 mg/kg). After decapitation, the hearts or forebrains were quickly excised and placed into isolation solution (depending on method) at +4°C.

2.2. Isolation of heart mitochondria

Mitochondria were isolated from rat hearts, as described by Jacobus and Saks (Jacobus and Saks, 1982). The hearts were washed in cold isolation medium (see Solutions), quartered and minced by scissors and incubated 15 min with trypsin (2.5 mg/20ml). Mitochondria were isolated by different centrifugations (600g and 8000g for 10 min) of tissue homogenized in glass-Teflon homogenizer. Isolated mitochondria were washed three times with isolation solution and centrifuged 4000g for 5min

2.3. Isolation of synaptosomes and brain mitochondria

Synaptosomes and mitochondria were isolated from the forebrains as described by Booth and Clark (Booth and Clark, 1978). The forebrains of 4 rats were washed with isolation medium (see Solutions). The brain tissue was cut to small pieces and homogenized with glass-Teflon homogenizer. Homogenate was centrifuged 1300g for 3 min at 4°C. The supernatant obtained was further centrifuged at 17000g for 10 min at 4°C, producing the crude mitochondrial/synaptosomal pellet. Mitochondria and synaptosomes were separated by using gradient (Ficoll 12 and 7.5%) in sucrose medium by centrifugation 99000g for 30 min at 4°C. The pellet containing mitochondria and synaptosomes were in the second interphase. Both were collected, diluted with isolation medium and homogenized gently in a glass-Teflon homogenizer. Pellets were diluted to 60 ml and centrifuged 5500g for 10 min at 4°C. The final synaptosomal and mitochondrial pellets were suspended in 2 ml of Mitomed.

2.4. Cardiomyocyte isolation

The heart was cannulated by aorta and perfused with the aerated and filtered (0.45 μm) isolation medium (IM) medium (see Solutions) during 5 min with a flow rate of 15-20 ml/min. The heart was digested by perfusion of IM (0.03 mg/ml liberase Blendzyme I and nominally zero calcium) in closed system (flow rate of 5 ml/min). The end of the digestion was determined following the decrease in pressure measured by a manometer. After the digestion, the heart was placed into IM containing 20 μM free Ca^{2+} and inhibitors for proteases (4.7 μg/ml leupeptin, 0.56 mg/ml trypsin inhibitor and 2 mg/ml BSA). Cardiomyocytes were released mechanically by forceps. Cell suspension was let to sediment three times at room temperature. Cardiomyocytes were gradually transferred from isolation medium (with 20 μM free Ca^{2+}) into Mitomed (nominally zero Ca^{2+}) in three steps.

Cardiomyocytes were washed 3 times with the Mitomed solution. Isolated cardiomyocytes contained 60-90% of rod-like cells.

2.5. Skinned fibers and their ghost preparations

Skinned fibers were prepared according to the method described by Saks *et al*. (Saks *et al.,* 1998). Cooled hearts were cut into halves and muscle strips (3–5 mm long and 1–1.5 mm in diameter, 5–10 mg of wet weight) were cut carefully from endocardium of left ventricles along fiber orientation. The muscle fibers were separated from each other by using sharp-ended needles, leaving only small areas of contact. The fibers were incubated for 30 min with mild stirring in cold solution A (containing 50 μg/ml saponin) for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed three times with solution B for 5 min to remove completely all metabolites, especially trace amounts of ADP. Complete removal of ADP was seen from respiration recordings which showed very reproducible initial State 2 rates (designated as v_o), not sensitive to inhibition by atractyloside.

Ghost fibers were prepared from skinned fibers after washing with solution C and selective extraction of myosin with solution D, containing 0.8 M KCl (see Solutions).

2.6. Cell culture

HL-1 cells were kindly provided by Dr. W. C. Claycomb (Louisiana State University Health Science Center, New Orleans, LA, USA). HL-1 non beating cells were obtained as described before (Pelloux *et al.,* 2006). Beating (B) and non beating (NB) HL-1 cells were cultured at 37°C under 5% CO_2 in fibronectin gelatin coated flasks containing Claycomb medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.1 mM norepinephrine. The cells were detached by trypsinization and were washed 3 times by centrifugation (1000 rpm) at +4°C with Mitomed solution (see Solutions) supplemented with 2 mg/ml BSA.

2.7. Determination of the initial rate of MgATPase reactions

The role of mitochondria in rephosphorylating the ADP produced in ATPase reactions was estimated as a decrease in the flux through the external system of ATP regeneration (PEP–PK–LDH) before and after activation of the mitochondrial oxidative phosphorylation by addition of the respiratory substrates (5 mM glutamate and 2 mM malate). Skinned cardiac fibers, cardiomyocytes or HL-1 cells were incubated in a measurement medium complemented with 5 mg/ml BSA, 5 mM PEP, 20 U/ml PK, 20 U/ml LDH and 0.24 mM NADH at 25 ^{o}C the cuvette of spectrophotometer (Cary 50, Varian, USA). The changes in optical density at 340 nm were measured before and after addition of ATP as well as after subsequent additions of the respiratory substrates and 98 μM atractyloside. The reaction rate was estimated from the stable and linearly time-dependent portions of the recordings.

2.8. Determination of the kinetics of respiration regulation by exogenous or endogenous ADP (exogenous ATP)

The steady state rates of oxygen consumption by permeabilized isolated cardiomyocytes, brain synaptosomes, HL-1 (B and NB) cells, skinned and 'ghost' cardiac fibers were recorded as a decrease in oxygen concentration over time by using the two-channel high resolution respirometer (Oroboros Oxygraph, Innsbruck, Austria) or Yellow Spring Clark oxygen electrode at 25° C, solubility of oxygen was taken as 215 nmol per ml.

Mitochondrial respiration was analyzed in all cells after permeabilization with 25-50 μg/ml saponin. Solution B was used for cardiac fibers and supplemented with 5 mg/ml of BSA and different free Ca^{2+} concentrations.

To achieve the maximal stability of the respiratory parameters for the permeabilized cardiomyocytes and HL-1 cells, the previously used solution B (Seppet *et al*., 2001; Saks et al., 2001, Saks et al., 1998) was replaced by the Mitomed solution and supplemented with 4 IU/mL hexokinase, and 12 mM glucose in case of NB HL-1 cells. The use of this solution allowed us to avoid using high EGTA concentrations and serious problems related to the contaminations in the commercial preparations of EGTA (Miller and Smith, 1984).

Respiratory substrates used were 5 mM glutamate with 2 mM malate, 5 mM pyruvate with 2 mM malate or 10 mM succinate. Kinetics of activation of the mitochondrial respiration was studied by increasing successively the final concentrations of ADP or ATP.

2.9. Trypsin treatment

Treatment of skinned fibers with trypsin was performed by incubation of fibers for 5 min with 50 nM trypsin in solution B (in the absence of BSA) and followed by washing the preparation three times in solution B (with 5 mg/ml BSA). Isolated cardiomyocytes were permeabilized by saponin and washed 4-5 times with Mitomed solution. Permeabilized cardiomyocytes were incubated with different trypsin concentrations (0.025-1.0 μM) for 5 min at 4^{o}C. Trypsin was inhibited by addition of 2 mM trypsin inhibitor and 2 mg/ml BSA.

2.10. Confocal imaging of mitochondria

To detect the mitochondrial functional state at the level of the single mitochondrion, images were acquired and analyzed by fluorescent confocal microscopy. For imaging of mitochondrial localization, cardiac cells ($10\text{-}20*10^3$ cells per chamber) were placed in Lab-Tek® chambered cover glass (Nalgen Nunc, Rochester, NY) and loaded with MitoTracker® Green (0.2 μM, Molecular Probes, USA) or MitoTracker® Deep Red (85 nM) and incubated at least 2 hours for cardiomyocytes at 4°C and 15 min – 2 hours for HL-1 cells at 36°C before analysis. Permeabilized synaptosomes were incubated with 100 nM of MitoTracker® Green, 45 min at 4°C in Mitomed solution.

To analyze mitochondrial inner membrane potential, cells were incubated for 30 min at room temperature with 50 nM tetramethylrodamine methyl ester (TMRM), a fluorescent dye that accumulates in mitochondria on the basis of their membrane potential. In control experiments, dissipation of membrane potential was observed after addition of 5 μM antimycin A, 4 μM FCCP, and 0.5 μM rotenone (data not shown). The digital images of TMRM and MitoTracker® Green fluorescence were acquired with inverted confocal microscope (Leica DM IRE2) with a 63-x water immersion lens. The MitoTracker® Green

fluorescence was excited with the 488 nm line of argon laser, using 510 to 550 nm for emission. TMRM fluorescence was measured using 543 nm for excitation (Helium-Neon laser) and greater than 580 nm for emission. A Mito Tracker® Deep Red flourescence was measured at 633 μm for excitation and 653−703 μm for emission.

Permeabilized cardiac fibers were fixed at both ends in a Heraus flexiperm chamber (Heraus, Hanau, Germany) by a microscope glass cover slide to analyze the intracellular mitochondrial distribution of myocytes or fibers and 200 μL of respiration medium was immediately added to the chamber. Myocytes or fibers were incubated for 30 min at room temperature with 50 nm tetramethylrhodamine ethyl ester (TMRE). Quantitative analysis of mitochondrial positioning in the cell was performed using the method developed by Vendelin et al. (Vendelin et al., 2005). The confocal images of the cardiac muscle fibers with easily distinguishable mitochondria preloaded with TMRE (50 nm) were used. Each image was rotated until the muscle fibers or cells long axis was oriented in a vertical direction, as judged by eye. Next, the centre of the mitochondria was marked manually, and the distances to the closest neighboring mitochondria were computed. The statistical analysis was performed by computing the distribution function of the distance between the centers of adjacent mitochondria (Vendelin *et al*., 2005).

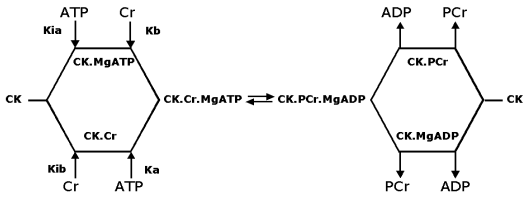
2.11. Creatine kinase

2.11.1. Total creatine kinase activity

Creatine kinase total activity in adult rat cardiomyocytes and cultured HL-1 NB cells was measured by UVVis spectrophotometer (Cary 50, Varian, USA) at 25°C in Mitomed solution (see Solutions) and contained additionally 2 mM ADP, 6 mM glucose, 0.6 mM nicotinamide adenine dinucleotide phosphate (NADP), 0.5 mM adenosine-5-phosphate, 2 U/ml hexokinase, 2 U/ml glucose-6-phosphate dehydrogenase. The rise in formed NADPH absorbance was measured after addition of 5 mM phosphocreatine (PCr) and total creatine kinase activity was calculated.

2.11.2. Kinetics of the creatine kinase reaction

The creatine kinase reaction follows a BiBi random type quasi-equilibrium reaction mechanism according to Cleland's classification (Cleland, 1963), with following kinetic scheme:



The dissociation constants of enzyme-substrate complexes for phosphocreatine (PCr) production are K_{ia}, K_a, K_{ib} and K_b. The K_{ia} and K_a are the dissociation constants of MgATP from binary complex CK.MgATP and ternary complex CK.Cr.MgATP, respectively. The K_{ib} and K_b are the dissociation constants of Cr from the binary complex CK.Cr, and from the ternary complex CK.Cr.MgATP, correspondingly. Following relationship is valid for quasi-equilibrium binding and dissociation of substrates (Jacobus and Saks, 1982): $K_{ia} \bullet K_b = K_{ib} \bullet K_a$

The values of above mentioned dissociation constants were experimentally determined in direction of phosphocreatine (PCr) production in isolated heart and brain mitochondria. Initial rates of MtCK reaction were measured in heart mitochondria because large part of kinetic data published about MtCK-ANT interaction are in the forms of calculated affinity constants, not the measured rates as it was needed for calibration of mathematical model. The initial rates of CK reaction in both mitochondria were measured in the presence (case I) and in the absence (case II) of the activated oxidative phosphorylation as described by Jacobus and Saks (Jacobus and Saks, 1982).

In the case I (Figure 11) the oxidative phosphorylation was activated in the presence of respiratory substrates (glutamate and malate for heart mitochondria and succinate and malate for brain mitochondria). In this case the respiration was maintained by production of ADP in the CK reaction. The kinetics of uMtCK and sMtCK activation in both isolated mitochondria was studied by respirometry in Mitomed solution at 30°C with gradually increasing concentration of ATP at different fixed concentrations of Cr. The rate of the forward creatine kinase reaction was calculated from the respiration rate, according to the equation (Jacobus and Saks, 1982):

$$V_{PCr} = 5.3 \times VO_2 \qquad (1)$$

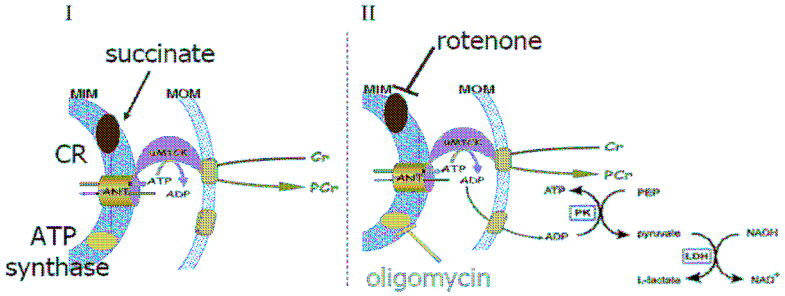


Figure 11. Schema about experimental protocol for investigation of the mechanism of functional coupling between MtCK and ANT. I: In the presence of the oxidative phosphorylation. II: In the absence of the oxidative phosphorylation, inhibition by oligomycin and rotenone.

In the case II (Figure 11), the oxidative phosphorylation was inhibited by oligomycin (1µM) as inhibitor of ATP synthase and rotenone (10 µM) as inhibitor of respiratory chain, and the rate of the MtCK reaction was measured in Mitomed solution with a coupled enzyme system (PEP-PK-LDH: PEP 5 mM, PK 5 U/ml, LDH 5 U/ml, NADH 0.150 mM) by increasing gradually concentration of ATP at fixed Cr concentrations. The rate of NADH oxidation was recorded at 30°C by spectrophotometer.

In both cases the experimental data were analyzed in a following way. The initial reaction rates of MtCK reaction with Bi-Bi random type quasi-equilibrium mechanism are described by the following equation (Jacobus and Saks, 1982):

$$V = \frac{Vm.[Cr].[MgATP]}{Kia.Kb + Ka.[Cr] + Kb.[MgATP] \quad [Cr].[MgATP]} \qquad (2)$$

For determination of the dissociation constants, the classical primary and secondary analysis by a linearization method was used (Jacobus and Saks, 1982; Saks *et al.*, 1985), instead of

popular fitting methods (Chen *et al*., 2000; Szafranska and Dalby, 2005), which application lacks the potential to illustrate the important information of the mechanism of the effects studied. For initial reaction rate measurements, the concentration of one substrate was fixed at different values and the rate determined as a function of the other substrate. For primary analysis, the measured reaction rates were expressed in double reciprocal plots as a function of the concentration of this substrate. If MgATP concentration was changed at fixed creatine concentration, in double reciprocal plots the reaction rate was expressed as:

$$1/v = \frac{1}{[MgATP]}\left(\frac{1}{Vm}\left(\frac{Kib\ Ka}{[Cr]} + Kia\right)\right) + \frac{1}{Vm}\left(\frac{Ka}{[Cr]} + 1\right) \tag{3}$$

If the creatine concentration was changed at different fixed MgATP concentrations, the following rate equation in double reciprocal plots was used:

$$1/v = \frac{1}{[Cr]}\left(\frac{Kb}{Vm}\left(\frac{Kia}{[MgATP]} + 1\right)\right) + \frac{1}{Vm}\left(\frac{Ka}{[MgATP]} + 1\right) \tag{4}$$

From these primary linear plots the ordinate intercepts i_o can be found as a linear function of the reciprocal of the concentration of the first substrate, which allows by the secondary analysis to find the values of the K_a, K_b and V_m, respectively, in the following way. From equation (3) we have:

$$io = \frac{1}{Vm}\left(1 + \frac{Kb}{[Cr]}\right) \tag{5}$$

From equation (4) we have:

$$io = \frac{1}{Vm}\left(1 + \frac{Ka}{[MgATP]}\right) \tag{6}$$

Secondary analysis of i_o vs 1/[MgATP] gives in the first case $-1/K_b$, in the second case it gives $-1/K_a$.

Accordingly, analysis of the slopes from the primary linear analysis (equations 3 and 4) allows finding the value of K_{ia} and K_{ib} in the following way. From equation (3):

$$slope = \frac{Kb}{Vm}\left(\frac{Kib}{[Cr]} + 1\right) \tag{7}$$

From equation (4):

$$slope = \frac{Kb}{Vm}\left(\frac{Kia}{[MgATP]} + 1\right) \tag{8}$$

From the abscissa intercepts of these dependences the values of $1/K_{ib}$ and $1/K_{ia}$ can be found.

It is important to note that these equations will be different when the reaction mechanism becomes the ordered one instead of random type (Segel, 1975). Thus, the secondary analysis gives immediately the information of the reaction mechanism.

2.12. Solutions used throughout the work

Composition of the solutions used was based on the information of the ionic content in the muscle cell cytoplasm (Godt and Maughan, 1988).

Solution A, in mM: CaK_2-EGTA, 1.9; K_2-EGTA, 8.1; $MgCl_2$, 9.5; DTT, 0.5; K-MES, 50; imidazole, 20; taurine, 20; Na_2ATP, 2.5; PCr, 15; pH 7.0

Solution B (with 0.1 μM free Ca^{2+}) in mM: CaK_2-EGTA, 1.9; K_2-EGTA, 8.1; $MgCl_2$, 4.0; DTT, 0.5; K- 100; imidazole, 20; taurine, 20; K_2HPO_4, 3; 5mg/ml BSA, pH 7.1. Solution B with different free Ca^{2+} concentrations (0.2–4.0 μM) was made by adding CaK_2-EGTA and K_2-EGTA stock solutions in different ratios calculated by Winmaxc program.

Solution C (washing solution for ghost preparation) contained, in mM: K-HEPES, 50; $MgCl_2$, 10; ATP, 10; DTT, 0.5; taurine, 20; K-Mes, 80; (pH 7.1).

Solution D (myosin extraction solution) contained, in mM: KCl, 800; K-HEPES, 50; $MgCl_2$, 10; ATP, 10; DTT, 0.5; taurine, 20 (pH 7.1).

Mitomed solution (Saks *et al.,* 1998) with following composition, in mM: sucrose, 110; K-lactobionate, 60; EGTA, 0.5; $MgCl_2$, 3; DTT, 0.5; taurine, 20; KH_2PO_4, 3; K-HEPES, 20; 2 mg/ml essentially fatty acid free BSA, pH 7.1. Mitomed was used for measurements.

Isolation medium for synaptosomes and brain mitochondria, in mM: sucrose 320; potassium EDTA 1; Tris-HCl 10; pH 7.4

Isolation medium for heart mitochondria, in mM: sucrose 300; potassium EDTA 0.2; K-HEPES, 10; pH 7.4

Isolation medium for cardiomyocytes (IM) with the following composition, in mM: NaCl, 117, KCl, 5.7; $NaHCO_3$, 4.4; KH_2PO_4, 1.5; $MgCl_2$, 1.7; glucose, 11.7; Cr, 11; taurine, 20; PCr, 10; HEPES, 21.1; pyruvate, 2; pH 7.1.

2.13. Reagents

The reagents were purchased from Sigma, Roche and Fluka (Buchs, Switzerland).

2.14. Data analysis

The values in tables and figures are expressed as means ± SD. The apparent K_m for exogenous ADP and ATP were estimated by the Michaelis–Menten equation by the nonlinear least squares fit, applying simple weighting of the experimental data. Statistical comparisons were made by using Student's *t*-test and analysis of variance (ANOVA) (variance analysis and Fisher test), and $P<0.05$ was taken as the level of significance.

2.15. Mathematical modeling of diffusion restrictions in cardiac energetics

The mathematical model was used to calculate coupling of MtCK and ANT. The model is based on free energy profile of MtCK-ANT interaction and earlier model of energy transfer by Vendelin *et al*. (Vendelin et al., 2000). By formally treating a coupled system as a single complex and taking into account that transfer rates between different states of the complex are linear in respect to the complex states, highly efficient numerical algorithms can be used to simulate the kinetics of the coupled system. In our case, a thermodynamically consistent model of coupled mitochondrial creatine kinase and adenine nucleotide translocase was composed that can be applied for the analysis of energy transfer of the cell.

The coupled MtCK and ANT system was modeled by using the following principles: (i) the coupled system was treated as one enzyme; (ii) the states of the coupled system were composed as a combination of states of participating enzymes and proteins; (iii) each transformation between states of different enzymes or proteins participating in the coupled system can change the state of only one participating enzyme. The exception is the transformation involving transfer of substrates between enzymes; (iv) The rate of transformation is always linear in respect to the state of the coupled system; (v) the coupled system was assumed to be in stationary state which was determined by the metabolites (ATP, ADP); (vi) a system of linear ordinary differential equations were composed to find the relative concentration of coupled system in each of the states,. The steady-state of this system would be eigenvector of the ODE system matrix corresponding to eigenvalue 0.

3. RESULTS AND DISCUSSION

3.1. The importance of cellular organization in regulation of respiration (articles I; III, IV, VI)

The role of structural organization of cells in determining the mechanism of regulation of respiration and interaction between mitochondria and ATPases were investigated in brain synaptosomes and in cardiac cells with clearly different structural organization. These cardiac cells were skinned cardiac fibers and their ghost preparations, isolated adult cardiomyocytes and cultured HL-1 cells with two subtypes, characterized by distinct contractile activities, depending on growing conditions, beating (B) cells and non beating (NB) cells.

Confocal fluorescent microscopy images of mitochondrial arrangement and intracellular organization in different cells showed very clear and remarkable differences between investigated cells (Figure 12). These images of isolated adult cardiomyocytes (Figure 12A) showed very regular arrangement of mitochondria, which followed crystal-like pattern with regular distances between neighbor mitochondria as it has been demonstrated by Vendelin *et al.* (Vendelin *et al.,* 2005). This regular arrangement of mitochondria, in parallel rows, is also preserved in ghost preparations after extraction of myosin from cardiomyocytes (Figure 12B) with persisted cell size and shape. The arrangement of mitochondria is intermediate in cultured HL-1 B cells (Figure 12D), which have some clustered dynamic mitochondria, but also some filamentous mitochondria with decreased mobility in parallel rows, most probably between some residual sarcomeres (due to beating properties). In contrast, absolutely chaotic and dynamic arrangement of filamentous mitochondrial network was observed in HL-1 NB cells (Figure 12D). Thus, the arrangement of mitochondria in HL-1 B cells were somewhere between adult cardiomyocytes and cultured HL-1 NB cells. Abundant mitochondria had granular appearance and rather fixed position in brain synaptosomes (Figure 12E).

Remarkable differences in the kinetics of respiration regulation by exogenous ADP and channeling of endogenous ADP (between these cells) were also observed in cardiac cells with different mitochondrial and cellular organization. High apparent K_m for exogenous ADP (in the range of 300-500 μM, see Table 2) was measured in isolated adult cardiomyocytes as it has been found in cardiac skinned fibers in many laboratories (Saks *et al.,* 1991, 1998, 2001; Seppet *et al.,* 2001). This high apparent K_m was preserved also in ghost cardiac fibers ~400 μM (Figure 15) as it has been reported previously by Andrienko *et al.* (Andrienko *et al.,* 2003). In permeabilized HL-1 NB cells, on the contrary, a very high sensitivity of mitochondrial respiration for exogenous ADP was observed — the value of apparent K_m for exogenous ADP (25 ± 4 μM), which is close to the value in isolated mitochondria and means that local concentrations of ADP near ANT does not significantly differ from those in surrounding medium, showing the absence of significant diffusion restrictions in these cells. The value of apparent K_m was intermediate in normally cultured HL-1 B cells (46 ± 15 μM) and in synaptosomes (110 ± 11 μM) showing that differences in respiration regulation were in good correlation with complexity of organization of mitochondria in these cells. This emphasizes the importance of structure in regulation of mitochondrial respiration. The data obtained are summarized in Table 1.

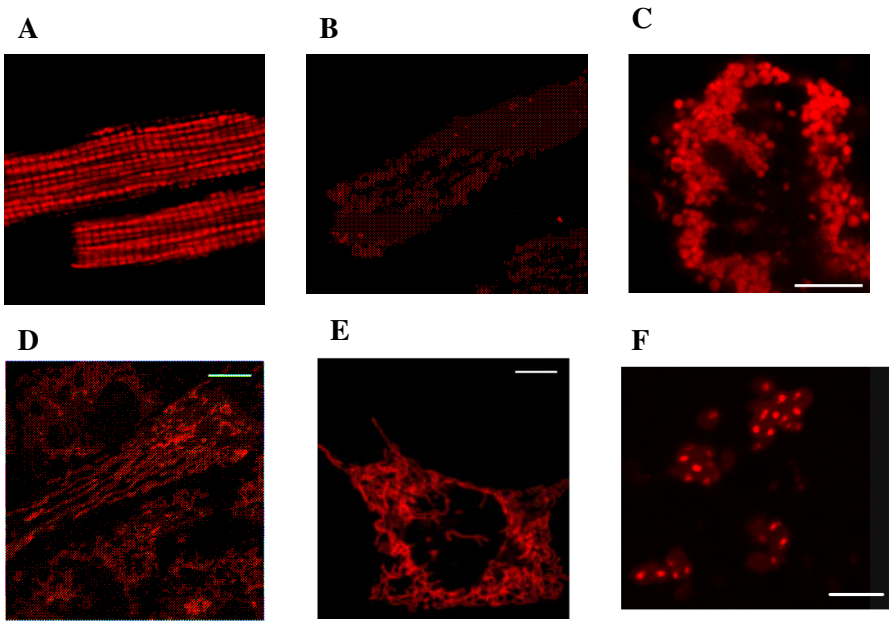


Figure 12 Differences in mitochondrial organization and localization in different cells. A isolated adult cardiomyocytes; B, ghost cardiomyocytes; C, HL-1 B cells; D, HL-1 NB cells; E, brain synaptosomes. Scale bars: C , D and E are 10 μm and F is 6 μm.

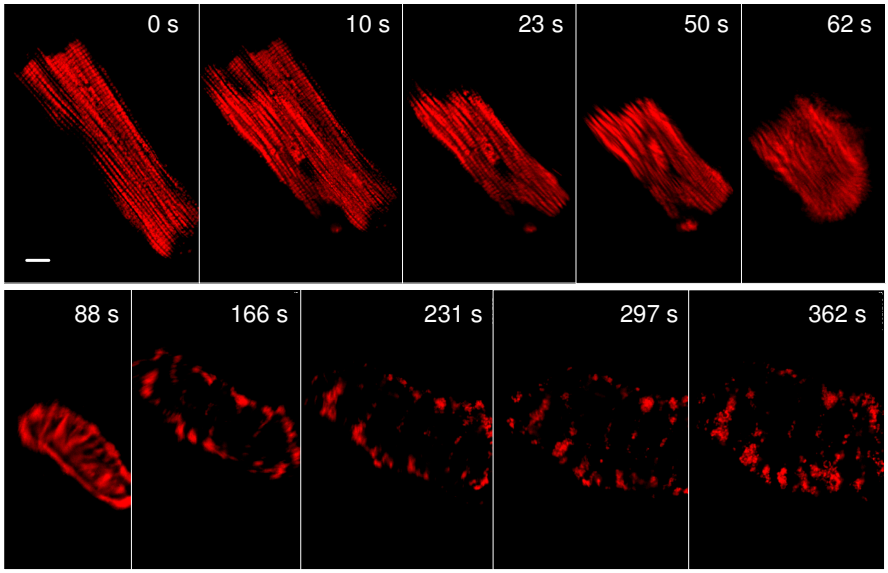


Figure 13 Dramatic changes in cardiomyocyte morphology and regular arrangement of mitochondria during cell incubation with saponin (50 μg/ml) and trypsin (1 μM). Mitochondria were visualized with TMRM (see Materials and methods). Confocal images of the same cell were taken successively at time points shown in seconds, s. Scale bar, 10 μm.

Table 1 Arrangement of the mitochondria and respiration regulation in different cells

Cells	Mitochondrial organization	K_m(ADP),μM	Reference
isolated adult cardiomyocytes	very regular in parallel rows with cristal-like pattern	360 ± 51	Fig. 1 article IV Fig. 4A, article VI
ghost cardiac cardiomyocytes	very regular distribution in parallel rows	350-400 (ghost fibers)	Fig. 2A, article I
synaptosomes	granular and fixed distribution	110 ±11*	Fig.4, article VI
B HL-1 cells	clustered dynamic; some are in parallel rows with decreased mobility	46 ± 15	Fig. 1A, article III
NB HL-1 cells	chaotic dynamic and filamentous mitochondrial network	25 ± 4	Fig. 1B, article III
isolated mitochondria	separated mitochondria	~17	Saks *et al.,* 1991

* - unpublished results

The high value of the apparent K_m in the regulation of mitochondrial respiration in permeabilized cardiomyocytes has been quantitatively explained by local diffusion restrictions for exogenous ADP in the level of the mitochondrial outer membrane and within the organized intracellular energetic units (Seppet *et al.,* 2001; Saks *et al.,* 2001, 2003; Vendelin *et al.,* 2004a). Highly organized cellular structure with regular arrangement of mitochondria in cardiomyocytes has been related to the interactions between mitochondria and some unknown trypsin sensitive cytoskeletal proteins (Kay *et al.,* 1997a). This very regular arrangement of mitochondria was completely lost in the result of treatment of cardiomyocytes with trypsin (Figure 12C). This process resulted in dynamic changes of cellular structure (Figure 13), probably due to the different sensitivity of cytoskeletal proteins towards trypsin and disequilibrium of elastic forces within the cell (Figure 13). Interestingly, mitochondria stayed attached to the cellular structures during the time of observation, (~6 min). Complete digestion of cardiomyocytes and liberation of mitochondria is achieved with higher trypsin concentration or longer incubation time.

The influence of these structural changes to the regulation of mitochondrial respiration was measured after short-time (5 min) incubation of cardiac cells with different trypsin concentrations (0.05 to 1.0 μM). Results on (Figure 14) shows significant decrease in the value of apparent K_m for exogenous ADP already after treatment with quite low trypsin concentrations (more than 0.05 μM). This shows clearly that diffusion restrictions for exogenous ADP decreased significantly with disorganization of cellular structure and regular arrangement of mitochondria after selective proteolysis of cardiomyocytes. Some

cytoskeletal proteins, responsible for regular mitochondrial arrangement and position to form highly organized structure of cardiomyocytes (and ICEUs), were digested and regular structure was lost. This disorganization in cellular structure of cardiomyocytes caused complete change in respiration regulation by exogenous ADP, showing that mitochondrial respiration regulation and cellular structure are closely related. At same time there was no effect on cellular structure or respiration regulation in either HL-1 cell subtype (results are not shown), demonstrating the absence of intracellular diffusion restrictions for exogenous ADP and showing surprisingly similarities between the behavior of mitochondria in HL-1 cells and isolated mitochondria.

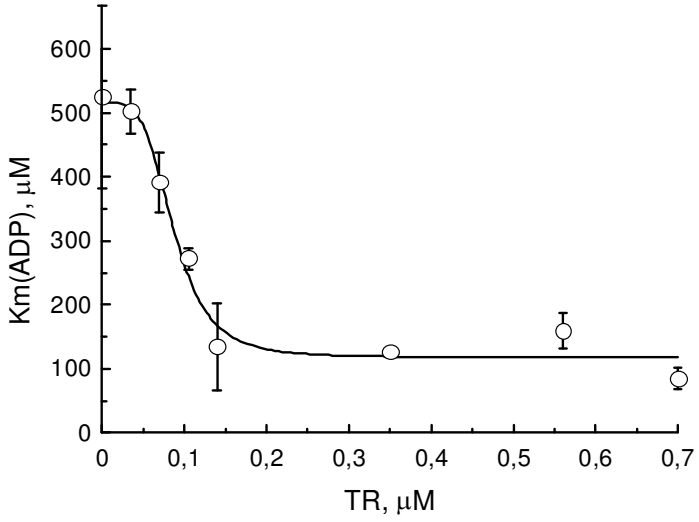


Figure 14 alteration of apparent K_m for exogenous ADP in respiration regulation of permeabilized cardiomyocytes after short term (5 min) treatment different trypsin concentrations

The localized diffusion restrictions *in vivo* are overcome by the effective phosphotransfer networks in the adult cardiomyocytes and oxidative skeletal muscles (Saks *et al.,* 2001; Saks *et al.,* 2006). The creatine kinase system is a major component of this network connecting mitochondria with ATP-consuming contractile system in myofibrils and ion pumps at the subcellular membranes (Saks *et al.,* 2001; Dzeja and Terzic, 2003). Direct channeling of endogenous ADP from MgATPases to mitochondria was demonstrated in isolated adult cardiomyocytes, when accessible ADP flux for PK-PEP-LDH system from MgATPases was significantly diminished after activation of oxidative phosphorylation (Figure 7A, article III). Most of the endogenous ADP was accessible for PK-PEP, but ~30% of endogenous ADP flux was directly channeled from MgATPases to mitochondria and this part of endogenous ADP was not accessible for competitive PK-PEP-LDH system in cardiomyocytes. In contrast to permeabilized cardiomyocytes, channeling of endogenous ADP was absent in the NB HL-1 cells, because not significant diminution in ADP flux was recorded after activation of mitochondrial oxidative phosphorylation (Figure 7B, article III), showing that mitochondria

were unable to capture ADP and compete with PK-PEP system. This indicates the absence of structural and functional interactions between mitochondria and ATPases in these cells.

The role of MtCK in regulation of mitochondrial respiration was investigated in cardiomyocytes and HL-1 cells after activation of mitochondrial respiration by endogenous ADP and trapping freely accessible endogenous ADP by competitive PK-PEP system (Fig. 8B, article III). Further activation of MtCK by addition of Cr in increasing concentration showed very strong activation of respiration in isolated adult cardiomyocytes. This result shows very important role of MtCK in regulation of respiration in cardiomyocytes and is clearly demonstrating functional coupling between ANT and MtCK. Activation of respiration in these conditions means that ADP produced locally by MtCK is not accessible to the powerful PK–PEP system, due to the limited permeability of the outer mitochondrial membrane (Fig. 8A, article III) and this ADP is directly channeled from MtCK to ANT and is further activating respiration in mitochondria. The apparent affinity of the coupled system for creatine is very high (apparent K_m is very low, 2.5 mM), which means that the maximal respiration rate is achieved at physiological creatine concentrations (Fig. 8C, article III). There was the absence of respiration stimulation by Cr was observed in NB HL-1 cells at same conditions (Fig. 9, article III). It is not yet clear whether the creatine kinase is totally absent or not active in these cells.

These results showed that diffusion restrictions and metabolic feedback regulation of respiration via phosphotransfer networks are most probably related to the organization of cellular structure.

3.2. Calcium-induced contraction of sarcomeres changes the regulation of mitochondrial respiration in permeabilized cardiac cells (articles I, II)

The relationship between cardiac cell structure and the regulation of mitochondrial respiration was investigated in cardiac cells with studying the influence of elevated free Ca^{2+} concentration on cardiac cell structure and mitochondrial respiration.

Strong contraction of intact permeabilized cardiac fibers was observed after addition of 1 μM free Ca^{2+} in the presence of 1 mM ATP (Fig. 1, article I) or, both, respiratory substrates (glutamate or pyruvate and malate) and ADP. The length of cardiac cell was reduced about 50% during these strong contractions caused by sarcomeres, as there was no contraction in ghost cardiomyocytes after removal of myosin (Fig. 2B,C, article I). This strong contraction observed was named `hypercontraction´ because of the absence of subsequent relaxation. However, as was shown, this strong contraction (Fig. 8A,B, article I) was reversible after transfer of fibers into solution B (see Materials and Methods for more details) containing 0.1 μM free Ca^{2+}, but no ATP and respiratory substrates (Fig. 8C, article I). Interestingly this strong contraction of sarcomeres was accompanied with remarkable, but reversible changes in respiration kinetics, apparent K_m decreased from ~300 to ~20 μM at 3.0 μM free Ca^{2+} (Fig. 8D, article I).

Analysis of the confocal images of the permeabilized cardiomyocytes and fibers with intact sarcomeres (Figs. 4 and 5, article I) showed that hypercontraction disorganized completely the localization of mitochondria within cardiac cells. The arrangement of mitochondria was analyzed in skinned cardiac fibers by using a quantitative method of image analysis of confocal micrographs developed by Vendelin (Vendelin *et al.,* 2005) based on measurement of distances between the centers of neighboring mitochondria in different

directions (along the fibers, in cross-fiber direction, in diagonal direction). The arrangement of mitochondria was analyzed in skinned cardiac fibers with intact sarcomeres, fixed at both ends (i.e. in isometric conditions), after addition of solution containing 3 μM of free Ca^{2+} in the presence of ATP, and compared to that in control solution containing 0.1 μM free Ca^{2+} but no ATP. The arrangement of mitochondria was changed from very regular crystal-like pattern (Vendelin *et al.,* 2005) in control solution (Fig 4B, article I) to chaotic distribution after elevation of free Ca^{2+} concentration up to 3 μM (Fig. 5B, article I). The average distance between mitochondrial centers was dependant from the direction between neighboring mitochondria, being largest in a diagonal direction, in control experiments with 0.1 μM free Ca^{2+} (Fig. 4C, article I). The average distance between mitochondrial centers was increased and independent from the direction between neighboring mitochondria after addition of 3 μM of free Ca^{2+} and in the presence of ATP (Fig. 5C,D, article I). This was considered a clear indication of remarkable change of the structure of cardiac cell.

It was shown that hypercontraction and disorganization of mitochondrial arrangement in cardiac cells in hypercontraction led to the strong alterations of the mitochondrial respiration kinetics (Figure 15) and channeling of endogenous ADP (see Fig. 9, article I) in the permeabilized cardiac fibers with intact sarcomeres. An increase of free Ca^{2+} concentration up to 4·μM decreased significantly the apparent K_m values of mitochondrial respiration for both, exogenous ADP and ATP (Figure 15B), from 320 to 20 μM and from ~300 to 54 μM, respectively. The strong effect of sarcomere contraction on the apparent K_m for both, exogenous ADP and ATP (Figure 15B) showed that structural connections between mitochondria and sarcomeres in the cardiac cells (inside ICEUs) were very significant. Ca^{2+}-dependent alteration of the structure of cardiac cells led to the changes of the regulation of mitochondrial respiration. Shortening of sarcomeres during Ca^{2+}-induced contraction of cardiac cells caused disintegration of the structures of ICEU and decreased the diffusion restriction for adenine nucleotides and adenine nucleotides became more easily available to mitochondria. Structural changes initiated by the contractile apparatus during hypercontraction led to the decrease of diffusion restrictions for adenine nucleotides. One of the possible explanations is that hypercontraction caused deformation of the mitochondrial outer membrane and opening of the VDAC to adenine nucleotides. Another possibility is that significant shortening of sarcomere length changes the structure of the ICEUs in unknown way and facilitates intracellular diffusion of exogenous ADP to mitochondria.

Activation of mitochondrial respiration by Ca^{2+} in cardiac fibers was not significant under these conditions. Maximal respiration rate (V_{max}) was not changed as much as the values of K_m during increase of free Ca^{2+} concentration (Figure 15A), as it showed only a moderate (50%) increase at lower Ca^{2+} concentrations, with an optimum at 0.4 μM free Ca^{2+} and decreased at higher Ca^{2+} concentrations. These changes in respiration rate were eliminated in case of ghost fibers (Figure 15C), which shows involvement of ATPases in this effect and shows clearly that calcium ions are unable to stimulate directly the respiration in cardiac cells without involvement of myofibrillar ATPases. This result favors the role of Frank-Starling relationship in regulation of cardiac muscle metabolism. Thus, parallel activation theory (Balaban, 2002) is probably not valid in cardiac cells.

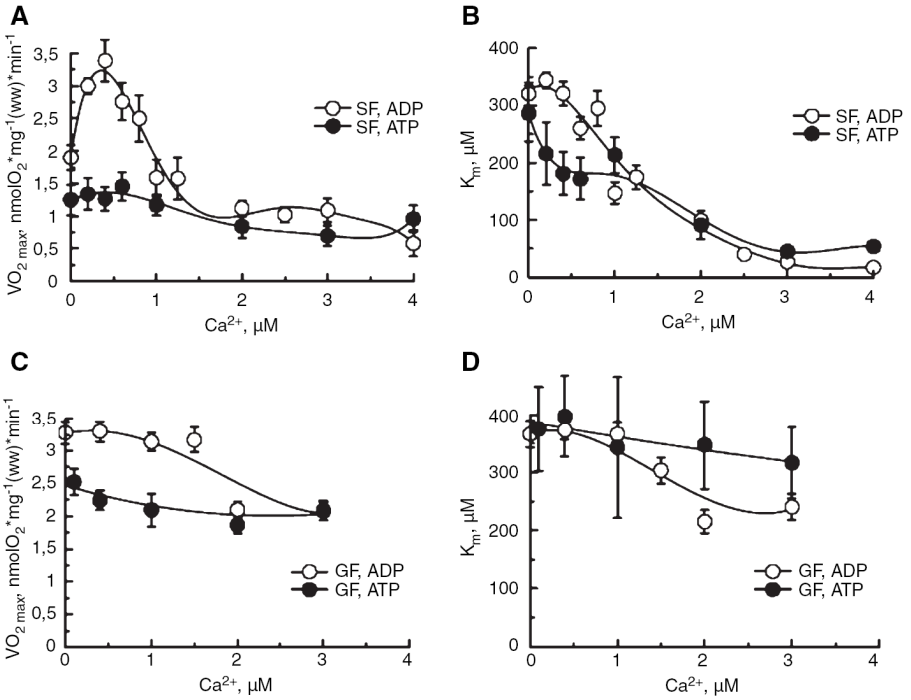


Figure 15 The effect of increasing free Ca^{2+} concentrations on respiration regulation in skinned and ghost fibers

The absence of contraction (Fig. 2 article I) and no statistically significant change in respiration kinetics (Figure 15C,D) in ghost fibers support the conclusion that Ca^{2+}-induced alterations in mitochondrial respiration is related to the changes induced by contraction of intact sarcomeres (or mediated from contractile apparatus) in permeabilized fibers and not induced by direct effect of Ca^{2+} on mitochondria. These results show that the direct effects of free calcium on mitochondrial respiration in cardiac cells *in situ* are not significant. This is consistent with other studies (Khuchua *et al.,* 1994), which have shown that there is no direct significant activation of mitochondrial respiration by Ca^{2+} ions in muscle cells in situ and the effects of changes in free calcium concentration rather result from indirect effects of the Ca^{2+} stimulation of actomyosin crossbridge cycling that provides ADP to activate respiration (Khuchua *et al.,* 1994).

Decrease in V_{max} of mitochondrial respiration in skinned fibers (Figure 15A), which resulted with an increase in concentration of free Ca^{2+}, was not caused by direct inhibitory effect of Ca^{2+} on ATP synthase as described by Holmuhamedov *et al.* (Holmuhamedov *et al.,* 2001), because activation of mitochondrial Na^+/Ca^{2+} exchanger by addition of Na^+ did not cause significant increase in respiration rate (Fig. 10, article I). Thus, a decrease in the V_{max} of respiration in skinned cardiac fibers is caused by hypercontraction but not by the direct effect of calcium on mitochondrial respiration. This is in concordance also with an insignificant decrease of V_{max} in ghost fibers during elevation of the free calcium concentration in the medium (Figure 15C).

It has been described in multiple studies that the apparent K_m for exogenous ADP in the regulation of mitochondrial respiration in skinned fibers can be effectively decreased by

short-term proteolytic treatment (Kuznetsov *et al.,* 1996). It was shown in this study that structural changes induced by Ca^{2+} had protective effect against proteolytic treatment — addition of calcium decreased the rate of the proteolytic degradation of proteins responsible for regular arrangement of mitochondria and for high apparent Km for exogenous ADP (Fig. 7, article I). This showed that ICEU structure was less accessible for trypsin, probably because of the more compact structure of cardiac cells due to the Ca^{2+}-induced hypercontraction.

The existence of direct channeling of ADP inside ICEU in cardiac cells has been shown by oxygraphic measurements in many studies with competitive system consisting of PK and PEP, which is consuming freely accessible ADP from the medium "ahead of mitochondria" (Fig. 3, article II). The influence of different free Ca^{2+} concentrations on endogenous ADP flux was studied spectrophotometrically (with coupled enzyme systems) in skinned cardiac fibers by addition of PK-PEP competitive system. The ADP available for this competitive system was measured spectrophotometrically and compared to that after activation of oxidative phosphorylation in skinned cardiac fibers. Scheme of this competitive system and idea of measurement is shown on Fig. 4 (article II). In the absence of oxidative phosphorylation (without respiratory substrates), the apparent K_m for MgATP in the MgATPase reaction was high (1.5–2.0 mM) in skinned fibers (Fig. 9, article I). This is explained by accumulation of ADP in the interior of the ICEU, and decrease in ATP/ADP ratio in myofibrils, which led to the inhibition of ATPases and thus, more ATP was needed to fully activate MgATPases. But after activation of the oxidative phosphorylation, by addition of respiratory substrates, the apparent K_m for ATP and the amount of ADP available for PK-PEP system were reduced significantly due to the direct channeling of endogenous ADP and increased turnover of both adenine nucleotides (Figure 15A,B). This means that PK-PEP competitive system was not able to consume endogenous ADP (diffusion of endogenous ADP out of ICEU is restricted as diffusion of exogenous ATP into ICEU), due to the coupling between ATP production and consumption by creatine kinase system. This direct flux of endogenous ADP from ATPases to mitochondria was diminished at high free-calcium concentrations (2 μM) (Fig. 9C, article I), compared to that at low calcium levels (0.1 μM) (Fig. 9B, article I) or at nominally zero Ca^{2+} (Fig. 9A, article I). Interestingly, the channeling of endogenous ADP was highest at 0.1 μM free Ca^{2+} (Fig. 9A,B,C, article I).

There are direct interactions between mitochondria and sarcomeres according to ICEU concept and changes in sarcomere length modify mitochondrial respiration. The activation of mitochondria keeps ATP/ADP ratio high in sarcomeres and is necessary for Ca-dependent crosstalk between mitochondria and sarcomeres (Kaasik *et al.,* 2001). The increase in the availability of endogenous ADP for PK-PEP system at high free Ca^{2+} (2 μM) concentration showed decreased diffusion restrictions for endogenous ADP and at same time diffusion barriers for exogenous ADP were also decreased, as explained earlier (Figure 15). Thus, hypercontraction, caused by high free [Ca^{2+}], disorganized the structure of ICEU in cardiac cells. The interactions between ATPases and mitochondria are assumed to regulate metabolism in cardiac cell via contraction-related alterations in localized diffusion restrictions for the intracellular diffusion of adenine nucleotides. This result is showing important role of the structure on respiration regulation and direct metabolite channeling in cardiac cells. Thus, existence of functional and structural relationship in cardiac cells was clearly demonstrated.

All of effects observed with an increase in Ca^{2+} concentration were unrelated either to mitochondrial calcium overload or to mitochondrial PTP opening (see control experiments on Fig. 3B,C, article I).

These results suggest that the structural changes transmitted from contractile apparatus to mitochondria by some unknown cytoskeletal proteins, which are responsible for ICEU structure, modify localized restrictions of the diffusion of adenine nucleotides and thus may actively participate in the regulation of mitochondrial function, in addition to the metabolic signaling via the creatine kinase system.

3.3. Thermodynamic and kinetic consideration of the coupled creatine kinase reaction in heart and brain mitochondria (articles V, VI)

The role and kinetics of the sarcomeric mitochondrial creatine kinase (sMtCK) reaction in isolated heart mitochondria and the ubiquitous mitochondrial creatine kinase (uMtCK) reaction in isolated brain mitochondria were studied to investigate the quantitative aspects of the mechanism of interaction of MtCK with oxidative phosphorylation and adenine nucleotide translocase (ANT). Heart mitochondria were used as reference for comparison of results from brain mitochondria and were also used for calibration of mathematical model for MtCK and ANT coupling developed by Vendelin, because most of published data about MtCK kinetics is mainly in the form of dissociation constants and not in intial rates as needed for mathematical modeling (Vendelin *et al*., 2007).

A complete analysis of the sMtCK reaction kinetics was carried out for isolated rat heart (Fig. 5, article V) and brain mitochondria (Figure 16 and Figure 17). Dissociation constants (K_{ia}, K_a, K_{ib} and K_b) were determined for the forward creatine kinase reaction for both substrates (MgATP and Cr) and conditions (in the presence and absence of oxidative phosphorylation) in heart and brain mitochondria (Table 2). The oxidative phosphorylation was inhibited by addition of inhibitors, olygomycin and rotenone, according to the simple kinetic protocol developed by Jacobus and Saks (Jacobus and Saks, 1982).

The complete kinetic analysis of MtCK reaction in both cases showed that the oxidative phosphorylation specifically altered only the dissociation constants for MgATP (K_a and K_{ia}) (see Table 2), by decreasing dissociation from the ternary complex CK.Cr.MgATP (Ka) and binary complex CK.MgATP (Kia). In particular, the values of dissociation constant K_a were decreased almost by an order of magnitude with activated oxidative phosphorylation in both, heart and brain mitochondria. Ka value decreased from 0.16 to 0.018 mM and from 0.13 to 0.018 mM in heart and brain mitochondria (Table 2). This means that the apparent affinity for this substrate (if Cr was already bound to MtCK) was increased by an order of magnitude. The dissociation constants for creatine (K_{ib}, K_b) were not changed under these conditions (see Table 2). These data show the tight functional coupling of uMtCK with ATPsupply from ANT in brain mitochondria, identical to that of sarcomeric sMtCK and ANT in cardiac mitochondria. The effects of the oxidative phosphorylation on the MtCK were identical in brain and heart mitochondria, showing similarities between mitochondria from cells with high metabolic rate. There was only slight difference in values for K_b (dissociation constant for Cr) between heart and brain mitochondria. The dissociation constants for creatine were somewhat lower in the case of uMtCK as compared to sMtCK (Table 2).

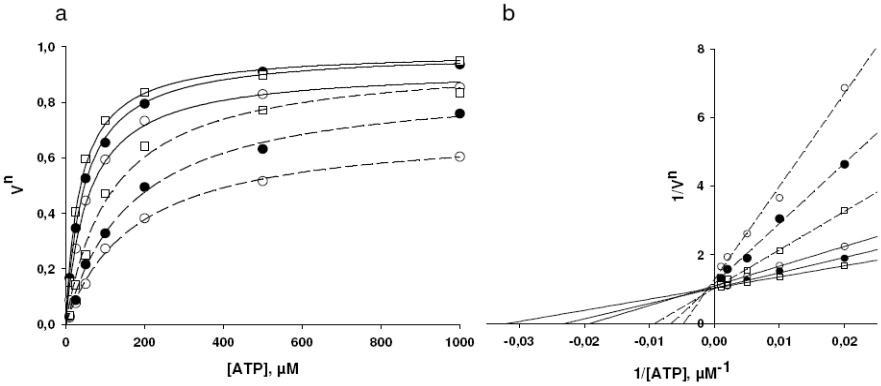


Figure 16 Primary normalized rate of CK reaction by exogenous MgATP and increased concentrations of Cr (5-10-15 mM) in the presence (solid lines) and absence (dotted lines) of oxidative phosphorylation (---). A: Michaelis-Menten representation of regulation of CK activity by exogenous MgATP B: Double reciprocal representation of regulation of CK activity by exogenous MgATP (Unpublished results)

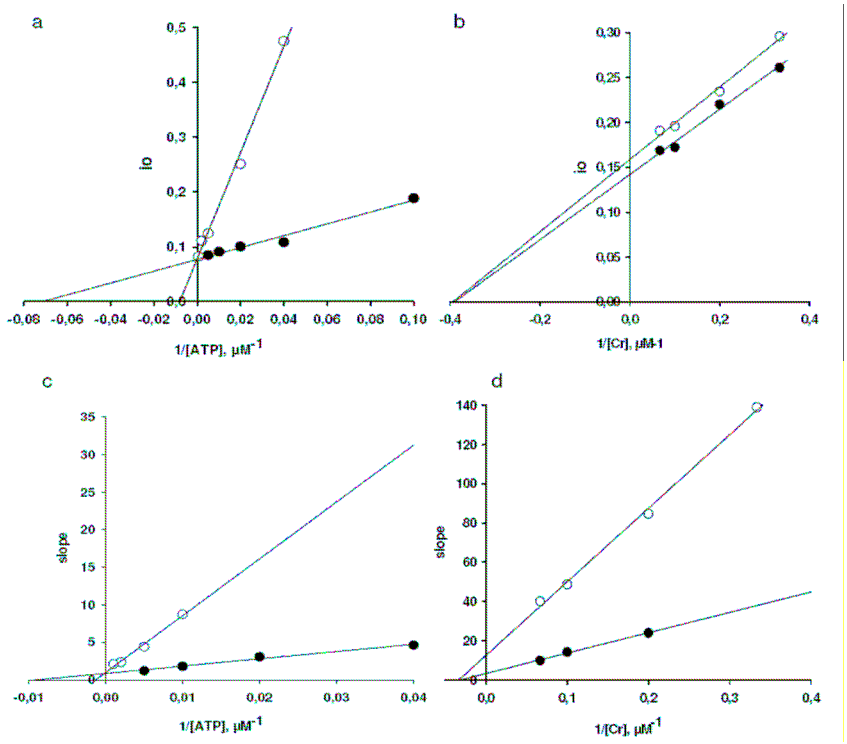


Figure 17 Secondary analysis of the data of primary analysis. A: The i_o of the lines plotted vs MgATP concentration and the values of abscissa intercept for determination of the dissociation constants for MgATP with and without oxidative phosphorylation. B: The slopes of the lines were plotted vs Cr concentration and the value of abscissa intercept allowed to determine the dissociation constant for Cr at both conditions: (●) with oxidative phosphorylation (o) without oxidative phosphorylation (Unpublished results)

Table 2 Comparison of the kinetic constants of the CK reaction in brain and heart mitochondria: the effect of oxidative phosphorylation (unpublished results)

Kinetic constants			Without oxidative phosphorylation	With oxidative phosphorylation
ATP constants:	Kia, mM	heart	0.85 ± 0.2	0.31 ± 0.1
		brain	1.1 ± 0,29	0.17 ± 0.07
	Ka, mM	heart	0.16 ± 0.04	0.018 ± 0.004
		brain	0.13 ± 0.02	0.018 ± 0.007
creatine constants:	Kib, mM	heart	34.7 ± 11	28.5 ± 2.6
		brain	24.8 ± 4	25.3 ± 5
	Kb, mM	heart	6.8 ± 3	4.4 ± 0.8
		brain	2.9 ± 1.1	1.7 ± 0.4

The difference in dissociation constants for MgATP demonstrates clearly that ATP produced in mitochondrial oxidative phosphorylation is more effective substrate for MtCK than exogenous MgATP from medium because of the direct transfer of ATP from ANT to the MtCK due to their spatial proximity which results also in increased uptake of ADP from MtCK (reversed direct transfer), and as a result, the turnover of adenine nucleotides is increased manifold at low external concentration of MgATP. This increased several times the rate of MtCK at low external concentrations of MgATP and maintains high rates of oxidative phosphorylation and PCr production in the presence of enough Cr. The MtCK reaction is strongly shifted in direction of PCr synthesis in spite of unfavorable kinetic and thermodynamic characteristics for this reaction. The Haldane relationship for the creatine kinase reaction was no more valid in case of activated oxidative phosphorylation, showing the involvement of oxidative phosphorylation and ANT in changing the kinetics of MtCK. This influence of oxidative phosphorylation for MtCK reaction demonstrates the existence of tight functional coupling between MtCK and ANT and the role coupled oxidative phosphorylation in alteration of MtCK kinetics. The oxidative phosphorylation itself controls the MtCK reaction and the PCr production in heart and brain mitochondria via ANT. At the same time, the MtCK plays back the same role for ANT and oxidative phosphorylation, by channeling ADP and thus directly controlling the rate of respiration. These data showed that the tight functional coupling of uMtCK with ANT in brain mitochondria was identical to that of sarcomeric sMtCK and ANT in cardiac mitochondria. In both cases the apparent decrease of dissociation constants of MgATP shows the effective cycling of ATP and ADP between MtCK and ANT.

The significant advantage and importance of the use of secondary analysis is the possibility to identify and directly illustrate the reaction mechanisms (Jakobus and Saks, 1982), in this case to characterize quantitatively and illustrate the mechanism of interaction between uMtCK, ANT and oxidative phosphorylation. Indeed, formally in both cases the reaction rates are described by the same type of equations (equation [2] and [3]), and the secondary analysis of the slopes shows that the reaction mechanism was always of random type with respect to substrates in the surrounding medium, and not converted into the ordered reaction mechanism (Cornish-Bowden, 2004). Active oxidative phosphorylation alters significantly only the dissociation constants of MgATP: most significantly the dissociation constant Ka from ternary complex CK**.**Cr**.**MgATP, but also, to some extent, the dissociation

constant Kia from binary complex CK**.**MgATP (Figure 17A, C, but does not change the dissociation constants for creatine (Figure 17B,D). Alternative approaches of directly fitting primary experimental data with proposed rate equation by using computer programs to find the values of kinetic constants (Chen et al., 2000) are rapid and convenient, but these methods usually leave researches in complete obscurity about real verification of reaction mechanisms (ordered, random type etc.). At the same time, classical secondary analysis (Cornish- Bowden, 2004) illustrates these mechanisms immediately, and shows the particularities introduced by interactions of enzymes with their partner proteins - in this case the mechanism of interaction of uMtCK with ANT. The plots of slopes versus reciprocal concentration of the second substrate (Figure 17C,D) are different for random type and ordered mechanism. Most importantly, the secondary analysis shown in Figure 17 directly and very clearly illustrates the mechanism of interaction between uMtCK and ANT – the specific and characteristic decrease of the dissociation constants for MgATP (apparent in this case for MgATP in the medium, for which it is calculated) clearly shows the direct channelling of ATP and rapid recycling of adenine nucleotides due to the high degree of structural organization of cardiac cells.

The kinetics of uMtCK in isolated brain mitochondria was analyzed in detail the first time in this study. The results of sMtCK kinetics in isolated kinetics isolated heart mitochondria were in a good accordance with those published by Jacobus and Saks (Jacobus and Saks, 1982).

The effect of oxidative phosphorylation on the kinetics of the creatine kinase reaction in heart mitochondria, particularly the decrease of Ka by more than an order of magnitude under these conditions was recently analysed by mathematical model developed and modified by Vendelin (Vendelin *et al*., 2000). The model based on thermokinetic analysis and free energy profiles of MtCK-ANT interaction (Vendelin et al., 2004b). First time direct channeling was analyzd as it is defined: a direct delivery of a reaction intermediate from the active cite of one enzyme to the active cite of a second enzyme without dissociation in to the bulk phase. This was achieved by treating the coupled system of ANT and MtCK as one enzyme. This analysis showed that oxidative phosphorylation specifically alters the free energy profile of the MtCK reaction by increasing the energy level of ATP in the complex with ANT under conditions of oxidative phosphorylation, thus making the PCr production thermodynamically favourable, contrary to the thermodynamics of the CK reaction in isolated state (Vendelin *et al*., 2004b).

The results of this study show that a similar mechanism is operative in the brain mitochondria. Thus, in heart, brain, skeletal and smooth muscle and some other cells, both ANT and MtCK function within a real proteolipid supercomplex with the ATP-synthasome and VDAC, thus connecting mitochondrial ATP production with the cytoplasmic reactions of energy utilization *via* MtCK and VDAC (Dzeja and Terzic, 2003). This conclusion is in excellent concord with important new data showing that due to the active functional coupling between MtCK and ANT, the MtCK induced ADP recycling strongly decreases the production of reactive oxygen species (ROS) in brain mitochondria (Meyer *et al*., 2006).

Analysis of experimental data of CK cardiac mitochondria with mathematical modeling confirmed the existence of PCr shuttle and showed also the importance of CK system in metabolic stability of cardiac cells. Mathematical modeling of the intracellular diffusion and energy transfer showed that the main function of the PCr–CK pathway is to transfer ATP from mitochondria to the different compartments for energy consumption and to activate

respiration by feedback signal of ADP and P_i. This energy transfer helps to overcome the local restrictions and diffusion limitation for adenine

Results of this study showed that the mechanism of functional coupling between uMtCK in brain mitochondria is identical to that seen with sMtCK and ANT in the heart. The detailed characterization of the kinetic behaviour of the uMtCK in isolated and purified rat brain mitochondria showed a tight functional coupling between this CK isoenzyme and ANT, and the role of this coupled system in the control of oxidative phosphorylation. These results conform the excistence of PCr-CK system in energy transfer in brain cells, and for the first time describe the presence of this system in the nerve endings – synaptosomes.

These results showed that direct transfer of ATP from ANT to MtCK is taking place in rat heart and brain mitochondria and the functional coupling of ANT and MtCK may be an important mechanism in energy transfer system in cells with high energy need. The coupling of MtCK and ANT reactions is essential for minimizing energy gradients, reducing energy dissipation, and directing energy to sites and pathways for specific processes (Saks et al., 2006; Dzeja and Terzic, 2005). Therefore, MtCK reaction was studied also *in vivo* (next chapter).

3.4. Integrated and organized cellular energetic systems in heart and brain (articles III, V, VI)

The importance of functional coupling between MtCK and ANT in heart mitochondria, isolated cardiomyocytes and cultured HL-1 cells was investigated by using an effective competitive enzyme system composing of PEP and PK, introduced by Gellerich *et al.* (Gellerich *et al*., 1982), to measure directly the channeling of ADP from MtCK to ANT. This competitive enzyme system let to follow the functional coupling of ANT and MtCK *in situ*, because it is trapping freely diffusing ADP and is competing with ANT for ADP.

At first, respiration was activated in cardiomyocytes by endogenous ADP produced by ATPases from exogenously added ATP (Figure 18). Further addition of powerful PK-PEP system caused inhibition of the respiration in cardiomyocytes. This means that most of the ADP produced by extramitochondrial ATPases or extramitochondrial creatine kinases was consumed by PEP-PK system. The respiration recovered and achieved higher rates after gradual additions of the Cr. This means that endogenous ADP formed by MtCK was not accessible for PK-PEP system due to the limited permeability of mitochondrial outer membrane (Saks *et al.,* 2003) and direct transfer of ADP from MtCK by ANT into the mitochondrial matrix (Vendelin *et al.,* 2004b) and further activation of respiration in mitochondria. The respiration rate was higher after addition of Cr because of increased turnover of adenine nucleotides in mitochondria due to direct channeling of ATP and ADP between ANT and MtCK. Interestingly, addition of Cr caused very strong activation of respiration (near to the maximal rate) already at millimolar concentrations in cardiomyocytes. This means that the apparent affinity of the coupled system for Cr was very high (low apparent K_m was determined, 2.5 mM) and increased in mitochondria of cardiomyocytes compared to the isolated heart mitochondria (Table 2). Strong increase in apparent affinity of the coupled system (MtCK and ANT) for Cr in cardiomyocytes is obvious from comparison of apparent K_m for Cr (2.5 mM) in cardiomyocytes with the dissociation constants for Cr in MtCK reaction determined in isolated heart mitochondria (K_{ib} ~ 28.5 mM and K_b ~ 4.4 mM, Table 2). This shows that regulation of respiration was

altered by functional coupling between ANT and MtCK in cardiomyocytes compared to isolated mitochondria. Increased affinity for Cr in MtCK reaction in cardiomyocytes shows probable change of dissociation constants for both MtCK substrates (ATP and Cr). Further studies are still needed for complete quantitative analysis of the sMtCK reaction mechanism in cardiomyocytes *in situ*, but the results obtained in this work showed that MtCK may have important physiological role in regulation of the mitochondrial respiration.

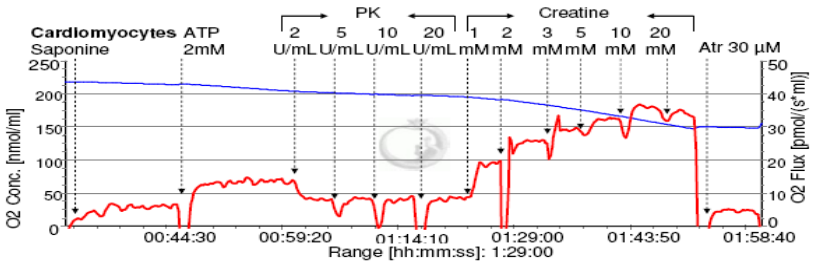


Figure 18 Respiration in cardiomyocytes with PK-PEP system

The experiment with PK-PEP system in isolated cardiac mitochondria was completely different from isolated cardiomyocytes. Almost complete inhibition of the respiration was observed in isolated heart mitochondria after addition of the competitive PK-PEP system into the medium (Figure 19). The addition of Cr was activating respiration in isolated mitochondria without the competitive PK-PEP system, which shows the existence of MtCK activity (Figure 20). This means that most of the ADP formed by ATPases (which always accompany isolated mitochondria in some extent) was freely accessible for the PK-PEP system. Further addition of Cr caused activation of respiration at smaller extent than in isolated cardiomyocytes. This means that ADP produced by MtCK reaction became to be available for PK-PEP system, which means that some ADP produced by MtCK was escaping from mitochondrial inner membrane space by VDAC due to the "leaky" mitochondrial outer membrane. This may be explained by the altered outer membrane of isolated mitochondria and loss of some important factors connecting mitochondrial outer membrane with highly organized intracellular structure and which are regulating permeability of the outer mitochondrial membrane. Therefore the alterations in MtCK kinetics *in situ* are most probably caused by highly organized cellular structure and connections with mitochondrial outer membrane and other cellular structures, which created diffusion restrictions and are lost during isolation of mitochondria.

Importance of the MtCK in regulation of respiration has been well demonstrated in cardiac fibers: the apparent K_m for exogenous ADP decreased from ~300 to ~80 µM, with addition of 20 mM Cr to the measurements of respiration kinetics (Saks *et al.,* 1991). Strong effect of Cr (20 mM) on respiration kinetics was demonstrated in brain synaptosomes in this work: the apparent K_m for exogenous ADP decreased from 110 ± 11 µM to 25 ± 1 µM. This shows importance of the PCr–Cr system in the energy transfer in brain synaptosomes. This shows that Cr is a very effective regulator of respiration *in situ* and respiration is regulated besides ADP feedback signal also by Cr flux from ATPases back to mitochondria and the activity of MtCK controls completely the respiration in cardiomyocytes. This is well demonstrated in Figure 7C (article VI), where cardiac fibers are with PK-PEP system and

PCr (20 mM) and stimulating action of Cr on respiration is only slightly modified by the presence of PCr. This means that mechanism of respiration regulation is very effective under *in vivo* conditions in cardiac cells due to the direct transfer of energy.

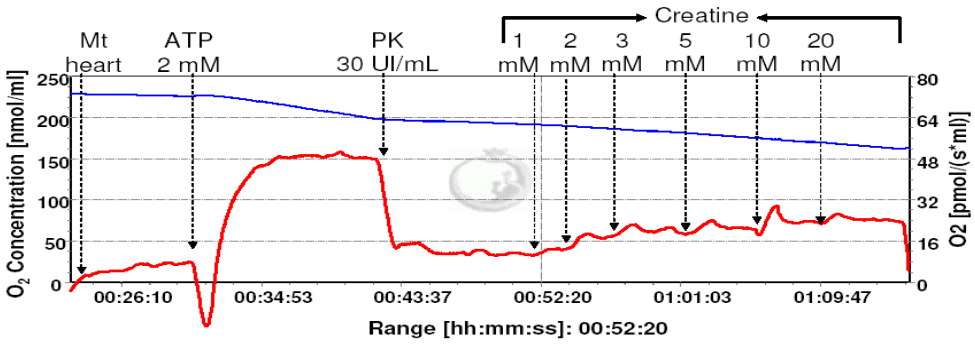


Figure 19 respiration recordings for isolated heart mitochondria with PK-PEP system. The respiration was activated by ADP produced by MgATPases from exogenous MgATP (2 mM). The addition of 30 IU/ml PK in the presence of 3 mM PEP removes most of this ADP. The activation of MtCK reaction by stepwise addition of Cr slightly increases the respiration of mitochondria (unpublised results)

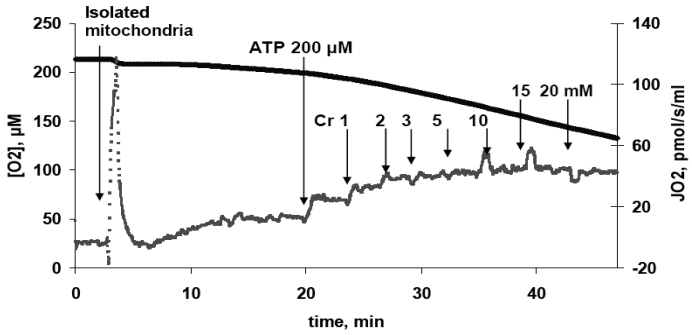


Figure 20 Oxygraph recording of the regulation of mitochondrial respiration by the uMtCK in isolated brain mitochondria. Respiration was activated first by 10 mM succinate and 200 µM ATP and then by increased concentrations of Cr as indicated (unpublised results)

Results of this study show that respiration regulation and the kinetics of the MtCK reaction is significantly dependent from cellular structure and highly organized cellular structure has direct influence on kinetics of the MtCK reaction. The functional coupling between ANT and MtCK is probably different and not comparable in mitochondria *in vitro* and in cardiomyocytes *in situ*. This difference may be the cause of some unknown cytoskeletal protein in mitochondrial outer membrane which forms diffusion barrier. Functional coupling in cardiomyocytes forms microcompartments for ATP and ADP in

mitochondrial inter membrane space due to this diffusion barrier on mitochondrial outer membrane, which is missing in isolated mitochondria.

The existence of some unknown protein component, which is responsible for this diffusion barrier was shown to be involved in isolated permeabilized cardiomyocytes (Figure 8G, article V) revealed by trypsin treatment. The gradual addition of PK at high activities (up to 100 U/ml) was not able to decrease respiration activated by endogenous ADP in intact cardiomyocytes, after activation of MtCK by 20 mM Cr at optimal concentration of free Ca^{2+} (0.4 μM). This means that mitochondrial outer membrane was intact and endogenous ADP was not accessible for PK-PEP system. Situation changed when regular mitochondrial organization was changed to chaotic by short incubation with trypsin. Possibly, some cytoskeletal protein which is responsible for positioning of mitochondria was digested by trypsin. Interestingly, this accompanied with opening of the MOM for ADP, and ADP escaped from mitochondria and PK-PEP system was effectively trapping it. This was demonstrating that the regulation of respiration is dependent on intracellular organization and mitochondrial arrangement. The same unknown factor (protein) may be responsible for forming contacts between mitochondria and cytoskeleton and might be responsible for diffusion restrictions and high apparent K_m for exogenous ADP (Saks *et al*., 2003; Vendelin *et al*., 2004a).

The effect of Cr on regulation of respiration in cardiomyocytes may be explained by regulation of the state of the voltage dependent anion channel (VDAC), possibly also by some unknown protein (Colombini, 2004) or by ANT-MtCK-VDAC complex formation (Rojo *et al.,* 1991; Brdiczka *et al.,* 1994; Biermans *et al.,* 1990). The latter makes transport of adenine nucleotides very efficient inside VDAC-MtCK-ANT complex: mitochondrial ATP is directly transferred from ANT to the MtCK and PCr produced by MtCK is transported through outer mitochondrial membrane by VDAC and there is no ADP diffusion out of mitochondria from intact cardiomyocytes. Therefore, formation of complex may regulate respiration in cardiomyocytes according to the physiological need, which maybe be regulated by feedback signal from ATPases to the mitochondria in the form of ADP and Cr. This complex mediates ADP and Cr feedback signal from MgATPases to mitochondrial matrix and activates oxidative phosphorylation in cardiac cells. This ANT-MtCK-VDAC complex is absent in mitochondria due to the altered outer membrane.

This analysis showed that oxidative phosphorylation specifically alters the free energy profile of the MtCK reaction by increasing the energy level of ATP in the complex with ANT under conditions of oxidative phosphorylation, thus making the PCr production thermodynamically favourable, contrary to the thermodynamics of the CK reaction in isolated state. The results of this study show that a similar mechanism is operative in the brain mitochondria. Thus, in heart, brain, skeletal and smooth muscle and some other cells, both ANT and MtCK function within a real proteolipid supercomplex with the ATP-synthasome and VDAC, thus connecting mitochondrial ATP production with the cytoplasmic reactions of energy utilization *via* MtCK and VDAC (Dzeja and Terzic, 2003). This conclusion is in concord with important new data showing that due to the active functional coupling between MtCK and ANT, the MtCK induced ADP recycling strongly decreases the production of reactive oxygen species (ROS) in brain mitochondria (76). They are also in concord with and explain the data by Dolder et al. (66) showing that the ADP recycling between ANT and MtCK due to functional coupling of these proteins inhibits the opening of the permeability transition pore.

All these results allow us to propose the following additions to the scheme of the processes of energy transduction in intact synaptosomes initially proposed by Nicholls (Nicholls, 2003): to add into this scheme the coupled uMtCK-ANT system and then the whole energy transfer process by the PCr-CK system, as shown in Figure 21. According to this scheme, mitochondrial ATP is used for phosphocreatine synthesis in the non-equilibrium uMtCK reaction after its direct transfer to the active center of this isoenzyme by ANT. At the plasma membrane of synaptosomes, PCr is used for local reproduction of the ATP. Another source of necessary ATP is the glycolytic system.

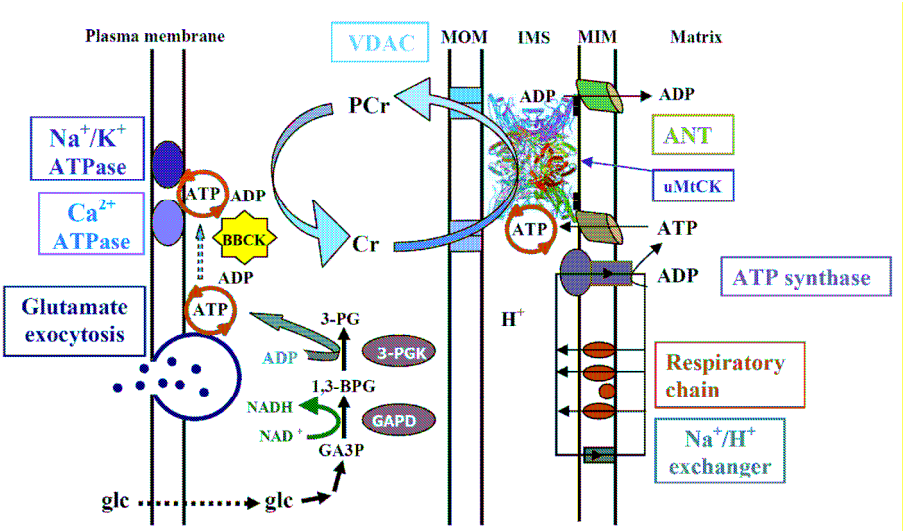


Figure 21 Energetics of brain synaptosomes. Sites of ATP production (mitochondrial matrix) and sites of ATP consumption (ion transport across the plasma membrane and vesicle trafficking for neurotransmitter uptake and release, e.g. glutamate) are linked by an energy transfer pathway represented by the phosphocreatine/creatine kinase system. uMtCK bound to mitochondrial inner membrane (MIM) via cardiolipin (black squares). ATP consumed by the energy consuming reactions is reproduced locally by BBCK from PCr. GA3P-glyceraldehyde-3-phosphate, 1,3-BPG-1.3 biphosphoglycerate, 3-PG-3-phosphoglycerate, GAPDH- glycerate-3-phosphate-deshydrogenase, 3-PGK-3-phosphoglycerate kinase (modified from Nicholls, 2003)

CONCLUSIONS

1. The apparent K_m for exogenous ADP measured was by more than an order of magnitude lower in the permeabilized HL-1 NB cells with dynamic and chaotic localization of filamentous mitochondria (25 ± 4 μM) in comparison with permeabilized adult cardiomyocytes (360 ± 51 μM), and intermediate in normally cultured HL-1 B cells (47 ± 15 μM). The apparent K_m for exogenous ADP was 110 ± 11 μM in brain synaptosomes. Low apparent K_m for exogenous ADP and the absence of the channeling of endogenous ADP characterized the HL-1 NB cells without significant diffusion restrictions. Higher apparent values of K_m for exogenous ADP were measured for more organized HL-1 B cells and brain synaptosomes. The data obtained supported the conclusion that the regulation of mitochondrial respiration and the energy transfer via energy transfer networks are related to the complex structural organization of the cells.

2. It was shown that addition of 1 or 3 μM free Ca^{2+} induced strong contraction of cardiac cells -´hypercontraction´, led to the complete disorganization of mitochondrial regular arrangement with the increase of average distance between adjacent mitochondria. The hypercontraction led to the significant change of the kinetics of mitochondrial respiration - the values of apparent K_m for endogenous ADP and ATP decreased from 320 μM to 20 μM and 300 μM to 54 μM respectively). The hypercontraction caused also a significant decrease in the flux of endogenous ADP channeled directly from MgATPases to mitochondria. The data obtained demonstrated that localized intracellular diffusion restrictions for adenine nucleotides were determined by high precise structural organization of the cardiac cell.

3. It was shown that selective proteolytic digestion of skinned cardiac fibers and permeabilized cardiomyocytes by trypsin caused remarkable disorganization of regular mitochondrial organization and decreased significantly the value of apparent K_m for exogenous ADP, from 300 to ~100 μM and from 500 μM to ~100 μM, respectively. This effect was most probably due to the digestion of some unknown cytoskeletal proteins, responsible for regular mitochondrial arrangement and localization in cardiac cells.

4. Dissociation constants for both substrates of the MtCK reaction (ATP and Cr) were calculated the first time for isolated brain mitochondria. Observed differences in the values of dissociation constants for MgATP in MtCK reaction with and without oxidative phosphorylation clearly demonstrated the functional coupling between ANT and MtCK in isolated brain mitochondria. The results obtained showed that ATP is channeled from ANT to uMtCK in brain mitochondria which indicated an important role of the PCr-CK system in energy transfer also in brain synaptosomes, similarly to heart mitochondria.

5. Strong control of the mitochondrial respiration by the MtCK reaction was demonstrated in isolated cardiomyocytes *in situ*. It was shown the first time that dissociation constants for MtCK reaction *in vitro* and *in vivo* are probably different indicating the importance of the intracellular organized structure in regulation of intracellular processes in cardiac cells.

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ABSTRACT

Integrated and organized cellular bioenergetic systems in heart and brain

Relationships between intracellular structure and regulation of mitochondrial respiration were studied in cells with high metabolic rate in this thesis. The role of mitochondrial creatine kinase in regulation of mitochondrial respiration and the importance of phosphocreatine-creatine system in energy transfer in brain and cardiac cells was also investigated. Confocal microscopy methods were used to study the organization and dynamics of mitochondria in cells with different cellular structure: different preparations of cardiac fibers, intact isolated cardiomyocytes, isolated nerve terminal preparations (so called synaptosomes), cultured HL-1 cells with two subtypes according to the contractile properties, beating and non beating cells (B and NB HL-1 cells, respectively).

Dependence of the respiration kinetics on intracellular positioning of mitochondria was investigated by respirometry methods. The absence of diffusion restrictions for adenine nucleotides was shown in NB HL-1 cells, with chaotic and dynamic mitochondria, and the value of apparent Km was very low, comparable to isolated mitochondria. The results showed existence of strict correlation between the respiration kinetics and complexity of cellular structure.

Alterations in cellular structure were initiated in cardiac cells by selective proteolytic treatment and elevation of free calcium concentration in medium to study relationships between cellular structure and respiration regulation. Proteolytic treatment of isolated cardiomyocytes changed very regular organization of mitochondria to chaotic with disappearance of diffusion restrictions for adenine nucleotides, showing that diffusion restrictions are related possibly to some protein which determines the position of mitochondria in cardiomyocytes. High free calcium concentration caused strong contraction which was accompanied by deformations in cellular structure and significant alterations in respiration kinetics. It was demonstrated that calcium alone is not sufficient to regulate respiration in mitochondria, but needs simultaneous activation of the sarcomeric MgATPases. The results obtained showed strict relationship between the cellular structure and function and may be used for further studies of the Frank-Starling law in cardiac cells.

Kinetic constants were determined for both substrates (ATP, Cr) in mitochondrial creatine kinase reaction in isolated heart and brain mitochondria. The kinetics of mitochondrial creatine kinase reaction was studied *in vitro* and *in vivo* in the presence or absence of oxidative phosphorylation. Tight functional coupling of mitochondrial creatine kinase and adenine nucleotide translocase was shown in isolated brain mitochondria due to the dependence of reaction kinetics on the presence or absence of oxidative phosphorylation. The central role of mitochondrial creatine kinase in regulation of respiration and energy transfer by phosphocreatine-creatine system was demonstrated in synaptosomes and cardiomyocytes *in vivo*. Differences in the kinetics of mitochondrial creatine kinase reaction *in vitro* and *in vivo* were demonstrated in cardiomyocytes, showing the role of highly organized cellular structure in this process.

The results obtained showed that cellular structure and the regulation of respiration are tightly coupled in cells with high metabolic rate, such as brain and heart cells.

KOKKUVÕTE

Integreeritud ja organiseeritud bioenergeetilised süsteemid südame- ja ajurakkudes

Käesolevas dissertatsioonis uuriti kõrge metaboolse aktiivsusega rakkude struktuuri ja mitokondrite hingamise regulatsiooni vahelisi seoseid – mitokondriaalse kreatiinkinaasi rolli hingamise regulatsioonil ning fosfokreatiin-kreatiin süsteemi osa energia transpordil südame- ja ajurakkudes.

Konfokaalmikroskoopia meetodite abil uuriti mitokondrite paiknemist ja dünaamikat erineva struktuuriga rakkudes: intaksetes isoleeritud südamerakkudes, aju närvilõpmete fragmentides (nn. sünaptosoomides), immortaliseeritud südamerakkude HL-1 rakukultuuri kahe alatüübi, kontraheeruvates ja mittekontraheeruvates rakkudes (B ja NB HL-1 rakkudes), ning südame lihaskiudude erinevates preparaatides. Kasutades oksügraafilisi meetodeid uuriti erinevate rakkude ADP-aktiveeritud hingamise kineetika sõltuvust rakkude ülesehituse organiseeritusest. Näidati, et kaootiliselt paiknevate ning liikuvate mitokondritega NB HL-1 rakkudes puuduvad difusioonitakistused adeniinnukleotiididele ning näiline K_m hingamise regulatsioonil on madal. Tuvastati selge korrelatsioon mitokondrite hingamise regulatsiooni ja rakkude struktuuri organiseerituse vahel.

Rakkude struktuuri ja hingamise regulatsiooni vaheliste seoste uurimiseks kutsuti uuritavates rakupreparaatides esile struktuurseid muutusi selektiivse proteolüüsi abil ning vaba Ca^{2+} kontsentratsiooni tõstmisega. Proteolüütiline töötlemine muutis regulaarse mitokondrite paiknemise korrapäratuks koos difusioonitakistuste kadumisega isoleeritud kardiomüotsüütides, mis näitas difusioonitakistuste võimalikku seotust mingi seni tundmatu mitokondrite regulaarset paiknemist määrava valguga. Vaba Ca^{2+} kontsentratsiooni tõstmine kutsus südamerakkudes esile tugeva kontraktsiooni, mis deformeeris südamerakkude regulaarse struktuuri, millega kaasnesid olulised hingamise regulatsiooni muutused. Näidati, et hingamise regulatsiooniks südamerakkude mitokondrites ei piisa ainult Ca^{2+} kontsentratsiooni tõstmisest vaid on vajalik ka samaaegne MgATP-aaside aktiveerimine. Saadud tulemused näitasid struktuuri ja funktsiooni vahelist tugevat seost ning andsid võimaluse täpsustada Frank-Starlingi seaduse rolli südamelihasrakkudes.

Määrati kineetilised konstandid mitokondriaalse kreatiinkinaasi reaktsiooni substraatidele (ATP, Cr) aju- ja südame isoleeritud mitokondrites. Mitokondriaalse kreatiinkinaasi reaktsiooni kineetikat uuriti nii *in vitro* kui ka *in vivo* paralleelselt südame-ja ajurakkude mitokondrites aktiveeritud ja inhibeeritud oksüdatiivse fosforüülimisega. Kreatiinkinaasi reaktsiooni substraatide kineetiliste konstantide muutumisega aktiveeritud oksüdatiivse fosforüülimise tingimustes näidati konjugeerituse olemasolu mitokondriaalse kreatiinkinaasi ja adeniinnukleotiidtranslokaasi vahel aju mitokondrites sarnaselt südame mitokondritele. Samuti näidati mitokondriaalse kreatiinkinaasi tsentraalset rolli hingamise regulatsioonis ning fosfokreatiin-kreatiin süsteemi tähtsust energia transpordil mitokondrite ja energiat tarbivate kompartmentide vahel nii südame- kui ka ajurakkudes. Isoleeritud südamerakkudes näidati mitokondriaalse kreatiinkinaasi substraatide kineetiliste konstantide erinevust *in vivo* ja *in vitro* tingimustes, mis tähendab, et kõrgelt organiseeritud südamerakkude struktuur mõjutab otseselt mitokondriaalse kreatiinkinaasi reaktsiooni kulgu.

Antud töös näidati, et kõrge metaboolse aktiivsusega rakkudes määrab intaknte kõrgelt organiseeritud struktuur energia transpordi efektiivsuse, tagades efektiivse oksüdatiivse fosforüülimise regulatsiooni ja ATP tootmise mitokondrites vastavalt ainevahetuse vajadusele, säilitades metaboolse stabiilsuse väga erinevates metaboolsetes seisundites.

ARTICLE I

Anmann, T., Eimre, M., Kuznetsov, A.V., Andrienko, T., Kaambre, T., Sikk, P., Seppet, E., Tiivel, T.,Vendelin, M., Seppet, E., Saks, V.A. 2005. Calcium-induced contraction of sarcomeres changes the regulation of mitochondrial respiration in permeabilized cardiac cells

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ARTICLE II

Seppet, E.K., Eimre, M., **Anmann, T.,** Seppet, E., Peet, N., Käämbre, T., Paju, K., Piirsoo, A., Kuznetsov, A.V., Vendelin, M., Gellerich, F.N., Zierz, S., Saks, V.A. Intracellular energetic units in healthy and diseased hearts

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ARTICLE III

Anmann, T., Guzun, R., Beraud, N., Pelloux, S., Kuznetsov, A., Ojeda, C., Tourneur, Y., Saks, V.A. 2006. Different kinetics of the regulation of respiration in permeabilized rat cardiomyocytes and in non-contracting HL-1 cardiac cells: importance of cell structure/organization for respiration regulation

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

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ARTICLE V

Saks, V., **Anmann, T.**, Guzun, R., Kaambre, T., Sikk, P., Schlattner, U., Wallimann, T., Aliev, M., Vendelin, M. 2007. The creatine kinase phosphotranspher network: thermodynamic and kinetic considerations, the impact of the mitochondrial outer membrane and modelling approaches

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ARTICLE VI

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2005	Tartu University, *Laboratory animal science: C-category competence course*
2003	Tartu University, *Practical course in confocal microscopy*
2001	Veterinary University, Food Toxicology Laboratory, Hannover, Germany, 2 month student exchange program IAESTE

Professional employment

2006 – .	National Institute of Chemical Physics and Biophysics, Scientist
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2003 – 2006	National Institute of Chemical Physics and Biophysics Engineer

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2005	Tartu Ülikool, *Mitokondrid lihasrakkudes: metodoloogilised aspektid ja düsfunktsioon südame-veresoonkonna haigustes*
2005	Tartu Ülikool, *Katselooma teadus: C-kategooria kursus*
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2003	Eesti Teaduste Akadeemia II auhind üliõpilaste parimatele teadustöödele

Teadustöö põhisuund

Kompartmentaliseeritud energia transpordi võrgustikud ja nende regulatsiooni mehhanismid kardiomüotsüütides

PUBLICATION LIST

1. Anmann, T., Eimre, M., Kuznetsov, A.V., Andrienko, T., Kaambre, T., Sikk, P., Seppet, E., Tiivel, T., Vendelin, M., Seppet, E., and Saks, V.A. (2005) Calcium-induced contraction of sarcomeres changes the regulation of mitochondrial respiration in permeabilized cardiac cells, *FEBS Journal 272*: pp. 3145–3161.
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