DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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INTERACTION OF MITOCHONDRIA AND ATPASES IN OXIDATIVE MUSCLE CELLS IN NORMAL AND PATHOLOGICAL CONDITIONS

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To my children with love

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ABBREVIATIONS

AF	atrial fibrillation
ADP	adenosine 5´-diphosphate
AK	adenylate kinase
AMP	adenosine 5´-monophosphate
AMPK	AMP activated protein kinase
ANT	adenine nucleotide translocase
AP_5A	diadenosine pentaphosphate
ASD	atrial septal defect
ATP	adenosine 5´-triphosphate
ATR	atractyloside
BB-CK	brain creatine kinase
BMD	Becker muscular dystrophy
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CK	creatine kinase
COX	cytochrome oxidase
Cr	creatine
Cyt	cytochrome
DMD	Duchenne muscular dystrophy
FAD	flavin adenine dinucleotide
$FADH_2$	1, 5-dihydro-flavin adenine dinucleotide
FCCP	carbonylcyanide-p-trifluorometoxy-phenylhydrazone
Fe-S	iron-sulphur
F-group	“Fibrillation” group
HF	heart failure
ICEU	intracellular energetic unit
IMS	intermembrane space
LDH	lactate dehydrogenase
MDX	dystrophin knockout
mi-CK	mitochondrial creatine kinase
MIM	mitochondrial inner membrane
MM-CK	muscle creatine kinase
MOM	mitochondrial outer membrane
mtDNA	mitochondrial DNA
NAD	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PEP	phosphoenol pyruvate
PK	pyruvate kinase
PLN	phospholamban

PTP	permeability transition pore
SERCA	sarcoplasmic reticulum Ca^{2+} -ATPase
SDH	succinate dehydrogenase
SID	superior-inferior distance
SR	sarcoplasmic reticulum
SR-group	“sinus rhythm” group
SRGR	succinate related glutamate respiration
UQ	ubiquinone
UQ H_2	ubiquinol
VDAC	voltage-dependent anion channel

INTRODUCTION

The mechanisms exerting control over oxidative phosphorylation in muscle cells *in vivo* are unclear. According to the classical concepts, the increased cytosolic [ADP] due to ATP splitting by ATPases is a main signal for respiratory stimulation (Chance and Williams, 1956, Chance et al., 1985). However, since the observations that myocardium responds to increased workload by linearly enhanced rate of oxygen consumption without fluctuation of cytosolic [ADP] (Neely et al., 1972, Balaban et al., 1986, Wan et al., 1993) this theory is not universally applicable anymore. Experiments using the saponin-permeabilized fibers have revealed that glycolytic fast-twitch muscles (e.g. *m. gastrocnemius*) display high apparent affinity to ADP in regulation of respiration (Km=10–20 μM) comparable to that in isolated mitochondria. In contrast, oxidative slow-twitch muscles such as heart and *m. soleus* exhibit a much lower affinity to ADP (Km=200–400 μM) (Kümmel, 1998, Saks et al., 1989, Seppet et al., 1991, Saks et al., 1995, Veksler et al., 1995, Kuznetsov et al., 1996, Liobikas et al., 2001). These results point to muscle type-dependent control over cellular respiration *in vivo*. Accordingly, it has been recently hypothesized that in oxidative muscle cells the mitochondria and ATPases form tight complexes, for convenience termed as the intracellular energetic units, ICEUs (Seppet et al., 2001, Saks et al., 2001). The ICEUs appear to compartmentalize a part of cellular adenine nucleotides so that they can be effectively used in specialized phosphotransfer networks (Seppet et al., 2001, Saks et al., 2001, Kaasik et al., 2001). The important feature of these networks is that they ensure effective stimulation of oxidative phosphorylation without significant changes in cytosolic adenine nucleotide and PCr contents, a condition termed as metabolic stability (Neely et al., 1972).

At present the structural and molecular nature of the ICEUs is unclear, neither is the existence of ICEUs proved in human myocardium. Observation that desmin-deficient mice exhibit the increased mitochondrial affinity to ADP in regulation of respiration in cardiac cells suggests the important role of cytoskeletal proteins in linking mitochondria and ATPase within one complex (Kay et al., 1997). However the studies addressing the role of other cytoskeletal proteins are yet to be performed.

This study addresses these problems by applying a complex set of techniques such as skinned muscle fibers, respirometry, kinetic analysis of enzymes, modification of genome, electronmicroscopy and laser confocal microscopy with special reference to muscle dystrophy and human heart disease. The results demonstrate the existence of the ICEUs in human atrial myocardium and show that within these units the mitochondria and ATPases are connected to each other via CK- and AK-phosphotransfer system, and by direct channeling of ATP and ADP. Secondly, the potential role of impaired oxidative phosphorylation and coupled to this AK and CK reactions in pathological conditions is

demonstrated. Thirdly, the importance of dystrophin in proper intracellular organization of the systems of energy metabolism is outlined. Fourthly, the study provides several new protocols to estimate the function of ICEUs intracellularly, by using the skinned fiber techniques. This approach has highly potential diagnostic value, since it can be used in assessment of small muscle specimen, e.g. the biopsy material.

REVIEW OF LITERATURE

The oxidative muscles (e.g. myocardium and *m. soleus*) are characterized by lower myosin ATPase activity, lesser glycogen content and glycolytic enzyme activities and higher activities of oxidative enzymes and larger content of mitochondria compared to glycolytic muscles (e.g. *m. extensor digitorum longus*) (Nemeth and Pette, 1981, Ogata and Yamasaki, 1997). Along with these metabolic characteristics the oxidative muscles also exhibit low rates of contraction force development that is explained by lower ATPase activity of type I myosin isoform, compared to that in type II isoform, typical for glycolytic muscle (Bárány, 1967). In cardiac cells the mitochondria occupy about 30% of the cardiomyocyte's space, whereas in *m. soleus* this percentage is much lower — 6%. High mitochondrial capacity explains why heart muscle produces more than 90% of ATP in the reactions of oxidative phosphorylation. In general the optimal energy metabolism in oxidative muscle cell relies on adequate capacity of mitochondria to produce ATP, effective transfer of energy from mitochondria to sites of its utilization (e.g. ATPases) and conversion of energy to work of contractile apparatus, ion pumps and protein synthesis system. Each of these steps can be controlled by multiple mechanisms to match the ATP production to its utilization in response to variable workloads.

1. Mechanisms of ATP production in mitochondria

Mitochondria represent the organelles which are capable of converting the energy released from oxidation of substrates into ATP, the universal energy source for ATPases, ATP-dependent ion channels, and contractile proteins. Mitochondria have an outer membrane (MOM) that is smooth and somewhat elastic and contains the voltage-dependent anion channel (VDAC) (also known as the mitochondrial porin) permeable for ions and metabolites with molecular weight up to 1500 D (LaNoue and Schoolwerth, 1984). The mitochondrial inner membrane (MIM) that has inward folds or invaginations called cristae, acts as a permeability barrier for a variety of compounds and contains the respiratory chain. The intermembrane space (IMS) is included between the two membranes. Inside the inner compartment is the matrix, a gel-like phase which contains the enzymes participating in Krebs cycle, mtDNA and ions. Mitochondria undergo dramatic changes in volume and state of organization during changes in respiratory activity (Scalettar et al., 1991, Leterrier et al., 1994, Mannella, 1982, 1994).

The mitochondria produce ATP mostly in reactions of oxidative phosphorylation. In this process the electrons generated from NADH or $FADH_2$ produced by oxidation of nutrients, such as glucose or fatty acids are transferred to molecular oxygen along the electron transport chain. The electron transport

chain consists of four respiratory enzyme complexes arranged in a specific orientation and sequence in a mitochondrial inner membrane. The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane and utilised by ATP synthase to make ATP from ADP and phosphate (Saraste, 1999).

The redox carriers within the respiratory chain consist of: flavoproteins, which contain tightly bound FAD or FMN as prostethic groups and undergo a ($2H^+ + 2\bar{e}$) reduction; cytochromes, with porphyrin prostethic groups undergoing a one-electron reduction; iron-sulphur (non-haem iron) proteins which possess prostethic groups also reduced in a one-electron step; ubiquinone (UQ), which is free, lipid-soluble cofactor reduced by ($2H^+ + 2\bar{e}$); and protein-bound Cu, reducible from Cu^{2+} to Cu^+.

1.1. Complex I (NADH-UQ oxidoreductase)

Complex I, or the NADH-ubiquinone oxidoreductase, is the largest of the three membrane bound enzymes that conserve energy in the mitochondrial respiratory chain by active transport of protons across the membrane. Complex I contains 42 or 43 different subunits in an unknown stoichiometry, one flavin mononucleotide (FMN), seven or eight different FeS centers, covalently bound lipid, and at least three bound quinol molecules (Walker, 1992, Friedrich et al., 1998). The monomeric complex I is over 900 kD, comparable in size to the protein component of the ribosome. Electron microscopy of single particles has revealed that complex I is an L-shaped structure with two major domains separated by the thin collar (Grigorieff, 1998). Complex I catalyses the transfer of two electrons from NADH to UQ this reaction being associated with proton translocation across the membrane with stoichiometry of $4H+/2\bar{e}$. Complex I can be inhibited by amytal, rotenone, piercidin A, demerol and mercurials that inhibit the electron flow from the Fe-S centers to UQ (Garrett and Grisham, 1995).

1.2. Complex II (succinate dehydrogenase)

The major component of this complex is succinate dehydrogenase (SDH), which occurs in all aerobic organisms as a membrane-bound enzyme of citric acid cycle. SDH is located on the matrix face of the membrane, feeds electrons from succinate to UQ and does not translocate protons (Hägerhäll, 1997). The SDH has four subunits, with that furthest from the membrane having a covalently bound FAD at the active site. A second peripheral subunit contains three Fe/S centres that provide a route for electrons into the membrane phase. The latter comprises two polypeptides, each of which contributes three α-

helices which sandwich two haem groups (Nicholls and Ferguson, 2002). Complex II can be inhibited by carboxin and thenoyltrifluoroacetone (Garrett and Grisham, 1995)

1.3. Complex III (bc1 complex or UQ-cytochrome c oxidoreductase)

Complex III consists of eleven subunits, but only three polypeptide chains carry the redox groups that are used in conservation of energy. These subunits are cytochrome b, FeS protein carrying a Rieske-type center (Fe_2S_2) and cyt c_1. Other subunits are small proteins that surround the metalloprotein nucleus, but two major proteins face the mitochondrial matrix and are homologous to mitochondrial processing peptidases, which function in protein import (Braun et al., 1995, Nicholls and Ferguson, 2002).

Complex III catalyses transfer of electrons from ubiquinol (UQH_2) to cytochrome c and couples this redox reaction to the generation of a proton gradient across the membrane by a Q cycle mechanism. Oxidation of quinols leads to active transport of protons across the membrane, which requires two active sites – one for the oxidation of UQH_2 and release of protons on the outer surface of membrane – Q_o site, also termed Q_p or Q_z, and one for the reduction of UQ coupled to the uptake of protons from the inner side of the membrane (Q_i or Q_n). This mechanism requires that electrons be transferred from the Q_o site to the Q_i site. The Q_o site for the oxidation of UQH_2 is located between FeS protein and cyt b, close to the cytoplasmic side of the inner mitochondrial membrane, and the Q_i site is in cyt b in the matrix side of the membrane (Xia et al., 1997; Iwata et al., 1998). Both sites are connected by channels. The two haemes of cyt b (b_H haem and b_L haem) have different redox potentials. The Q_o site is near to the high potential b_H heme.

The two electrons from quinol are transferred within the cyt bc_1 complex so that the first electron is transferred along a high-potential chain to the Rieske FeS center, and then to cyt c_1. The second electron is transferred to the Q_i site via the hemes b_L and b_H of the cyt b subunit. The two electrons are transferred to the Q_i site after oxidation of two quinols in the Q_o site, to reduce one quinone. This mechanism leads to a net translocation of four protons for each pair of electrons transferred to cyt c (Saraste, 1999). The Q-cycle can be inhibited by antimycin A at Q_n and myxothiazol at Q_p.

1.4. Cytochrome c and complex IV (cytochrome c oxidase, ferrocytochrome: O_2 oxidoreductase)

Complex IV is the third locus within the respiratory chain generating a transmembrane proton gradient. Complex IV contains 13 subunits (Tsukihara et al., 1996) among which subunit I contains the active site. Subunit II has a copper

center (Malmström and Aasa, 1993), which is the first site to receive electrons from cytochrome c. The substrate of cytochrome oxidase, cytochrome c, is a water-soluble hemoprotein that donates electrons on the cytoplasmic side of the mitochondrial inner membrane. These electrons are transported to the active site, which contains a haem iron and a copper, and they are used to reduce O_2 into two water molecules. The protons needed for this reaction are taken from the mitochondrial matrix side through two channels. The same channels are used to pump one proton per electron across the membrane. For each two electrons reaching an oxygen atom from cyt c, four protons are moved through the oxidase. Two of these protons can be regarded as pumped across the membrane but the other two protons coming from the N-side are used to meet the two electrons from the P-side. Thus, the proton pump stoichiometry is $2H^+/2\bar{e}$. Cytochrome c oxidase can be inhibited by cyanide, azide and carbon monoxide at the oxygen binding site. In addition, NO is a reversible inhibitor of complex IV, competing with oxygen (Nicholls and Ferguson, 2002).

1.5. ATP synthase

The mitochondrial ATP synthase (F_1F_0 ATPase or Complex V) is a functionally reversible enzyme — it can synthesize ATP using a protonmotive force across the membrane, but it also can hydrolyze ATP to pump protons against an electrochemical gradient. In aerobic conditions the function of the ATP synthase is to utilize Δp to maintain the mass-action ratio for the ATPase reaction 7–10 orders of magnitude away from equilibrium (Abrahams, 1994, Boyer, 1997). In the absence of oxygen, e.g. in ischemia the ATP synthase reaction reverses to produce the transmembrane proton gradient at the expense of glycolytically made ATP.

The ATP synthase enzyme is a complex protein. Part of the ATP synthase known as F_0, is embedded in the membrane and is connected by stalk-like structures to the the F_1 complex in a way that drives the ATP synthesis reaction (Ferguson, 2000). A membrane sector (F_0) contains the proton channel. It is linked to the catalytic component (F_1), located in the matrix side of the membrane. A soluble ATPase (F_1) can be detached from the complex, and it contains five different subunits — α, β, γ, δ , and ε- in a stoichiometry 3:3:1:1:1. The α and β subunits are homologous; both bind nucleotides but only β has catalytic activity. There are three active sites within the catalytic component (Saraste, 1999).

The question of how ATP is synthesized by F_1F_0 remains to be answered yet. The hypothesis of rotational catalysis (Boyer and Kohlbrenner, 1981, Cox et al., 1984) implies that the membrane sector contains a structure that rotates in response to protonmotive force. One candidate structure is an oligomer formed by subunit c, a protein present in 12 copies in F_0 (Jones and Fillingame, 1998).

Each subunit c contains a conserved carboxylic acid residue in the middle of the membrane bilayer. The protonation and deprotonation of this residue may be at the heart of the rotary mechanism. To ensure this mechanism, other components form a stator structure that opposes the rotor's movement. A key component of the stator is subunit a, which contains a conserved arginine that could counteract the moving glutamate in subunit c. The current model proposes that the dodecamer of subunit c forms the rotor with the γ and ε subunits, and the subunit a, b, and δ complex forms the stator arm. Proton movement through the interface between subunit a and the subunit c oligomer would cause a torque when the stator and rotor move in the opposite directions (Elston et al., 1998). As an outcome of the functioning of the rotary mechanism one ATP molecule is synthesized for 4 H^+ translocated through F_0.

The ATP synthase can be inhibited by oligomycin and dicyclohexylcarbodiimide that block proton conductance of F_0 (Nicholls and Ferguson, 2002).

Considering the stoichiometries of proton pumping and transfer of electrons to oxygen and ratio of protons required to synthesize 1 molecule of ATP the efficiency of oxidative phosphorylation can be calculated as follows: If the electrons originate from NADH, then the overall proton and charge stoichiometry for the transfer of 2ē is 10/2ē. However, the electron transfer from succinate (e.g. from UQ to oxygen) results in ratio of is 6/2ē. Correspondingly, the P/O ratio for NADH oxidation is 10/4 (=2.5), and for succinate 6/4 (=1.5). Oxidation of ascorbate at the level of cytochrome c oxidase results in P/O ratio of 1 (Nicholls and Ferguson, 2002).

2. The mechanisms of regulation of mitochondrial ATP synthesis

In normal conditions the processes of oxidative phosphorylation are regulated (i) by delivery of oxygen and substrates to the mitochondria, (ii) by availability of ADP and Pi, (iii) by intramitochondrial Ca^{2+} ions, and (iv) by biosynthesis of mitochondrial membranes. In the healthy human heart about 60–90% of the ATP generation in the mitochondria comes from β-oxidation of fatty acids, and 10–40% is derived from pyruvate, which is formed by glycolysis and from lactate. Because the fatty acid oxidation generates more $FADH_2$ relative to NADH than does glucose or lactate, the molar ratio of ATP to oxygen consumed is higher for glucose and lactate than for fatty acids. Therefore, oxidation of more fatty acids in lieu of glucose and lactate may worsen the mechanical efficiency of the heart muscle. Accordingly, the stable angina pectoris has been treated with the agents (e.g. trimetazidine) that directly suppress β-oxidation of fatty acids and stimulate PDH reaction thus improving the contractile efficiency of the heart (Stanley and Chandler, 2002).

The delivery of free fatty acids for mitochondria is mainly dependent upon their concentration in the plasma and the content of fatty acid transport proteins (fatty acid transport protein — FATP) and/or fatty acid translocase FAT/CD36 (Barger and Kelly, 2000) in the sarcolemmal membrane. In the cytoplasm the free fatty acids are esterified by long-chain fatty acyl-CoA synthetase with CoA to form long-chain fatty acyl-CoA. To pass the mitochondrial inner membrane, the long-chain fatty acyl moiety is converted to long chain fatty acylcarnitine by carnitine palmitoyltransferase I (CPTI) and subsequent translocase across the inner mitochondrial membrane is facilitated by carnitine/acylcarnitine translocase. CPTI reaction is a rate-limiting step in mitochondrial fatty acid flux, and, therefore, highly regulated at the transcriptional level (reviewed by Barger and Kelly, 2000). In addition it is regulated by reversible binding of malonyl-CoA that is the inhibitor of CPTI. In the matrix the long-chain acylcarnitines are re-esterified to acyl-CoA derivates by CPT II and enter the β-oxidation cycling. The cardiac expression of genes encoding mitochondrial fatty acid oxidation enzymes is coordinated by the transcription factor, peroxisome proliferator-activated receptor α (PPARα). In the heart and oxidative skeletal muscles activation of PPARα increases the expression of the following genes involved in fatty acid oxidation: (i) FATP, FAT/CD36, fatty acid binding protein, and acyl-CoA synthetase, (ii) CPT I/CPT 1β catalysing fatty acid mitochondrial import, and (iii) the enzymes of mitochondrial β-oxidation, such as medium-chain acyl-CoA dehydrogenases, long-chain acyl-CoA dehydrogenases and very long-chain acyl-CoA dehydrogenases (Barger and Kelly, 2000). There are several natural ligands that activate PPARα, such as eicosanoids (leukotriene B4, 8(S)-hydroxyeicosatetraenoic acid, and medium and long chain fatty acids. The ligand binding allows the PPAR with the retinoid X receptor to interact with coactivator proteins such as PGC-1 in the heart, and to bind to sequence-specific target elements (PPRE) in a promoter site of the target gene. This mechanism stimulates upregulation of enzymes of free fatty acid oxidation during postnatal development, after exercise and during fasting, as the physiological stimuli (Barger and Kelly, 2000).

In the heart the uptake of extracellular glucose is regulated by the transmembrane glucose gradient and the concentration and activity of the glucose trasporters GLUT 1 and GLUT 4, the latter predominating in the heart. Insulin and ischemia increase the translocation of GLUT 1 and GLUT 4 from intracellular sites to the plasma membrane. The overall rates of glucose uptake, glycogen synthesis and breakdown, and glycolysis are controlled by multiple steps at the level of phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, lactate transporter and pyruvate dehydrogenase (PDH) (Stanley and Chandler, 2002). Pyruvate oxidation via PDH is decreased by elevated rates of fatty acid oxidation. On the other hand, activation of carbohydrate oxidation at the level of PDH results in the inhibition of CPT I via elevated malonyl-CoA concentrations.

The studies of isolated mitochondria have revealed that the short term control over the rate of oxidative phosphorylation is shared between the respiratory complexes, ATP synthase, ANT carrier and substrate transporters. Therefore, the relative role of different factors is not constant but varies depending on the physiological conditions. For example, in state 4, when all available ADP has been transformed into ATP, the succinate-dependent respiration rate is predominantly controlled by passive proton leak through the inner mitochondrial membrane. A shift from state 4 to state 3 (after increasing availability of ADP and Pi) is associated with distribution of control between the ANT, the dicarboxylate translocator, the complex III and complex IV (Nicholls and Ferguson, 2002). The long term regulation of mitochondrial capacity of oxidative phosphorylation occurs via *de novo* synthesis of the components of the inner and outer mitochondrial membranes. Among them the uncoupler proteins have been shown to play important role (Nicholls and Ferguson, 2002)

3. The mechanisms of ATP utilization

3.1. Mechanism of excitation-contraction coupling and sarcomere contraction

Contractile activity is linked to two main events — changes in $[Ca^{2+}]$ as the source of activation and actomyosin ATPase activity as the chemio-mechanical transduction system. Together these processes form the mechanism of excitation-contraction coupling. In this process in cardiac cells, membrane depolarization triggers the transsarcolemmal influx of Ca^{2+}. This Ca^{2+} current then liberates larger amounts of Ca^{2+} from the SR through the so called Ca^{2+}-induced Ca^{2+} release mechanism, with participation of special channels — ryanodine receptors (Fabiato and Fabiato, 1975). As a result the intracellular (cytoplasmic) $[Ca^{2+}]$ sharply increases, registered as the ascending phase of the intracellular free Ca^{2+} transient. Increased intracellular $[Ca^{2+}]$ in turn leads to activation of actomyosin ATPase reaction and development of contraction. The following relaxation occurs due to active pumping of Ca^{2+} by Ca^{2+}-ATPases of SR into SR and by sarcolemmal Ca^{2+} –ATPase and Na^{+}/Ca^{2+} exchange out of the cells resulting in decrease in cytoplasmic Ca^{2+} levels (descending phase of intracellular Ca^{2+} transient) which accelerates dissociation of Ca^{2+} from troponin C. Under normal conditions 2/3 of the muscle cellular ATP is used for fueling contractile apparatus, while remaining 1/3 is used by ion pumps, primarily by SR Ca^{2+} -ATPase (Stanley and Chandler, 2002).

The actomyosin ATPase activity localizes in the myofibrillar compartment that amounts to about 60% of the mammalian cardiac cell volume (Barth et al, 1992). It is organized as a three-dimensional system of fibrillar proteins. The

contractile proteins form the myofilaments made of a series of contractile units — the sarcomeres, each of which consists of thick and thin filaments. Interaction between the thick and thin filaments leads to muscle contraction. The thick filament can be regarded as the location of the energy transduction system, consisting of myosin molecules bearing the ATPase activity on their two globular heads and forming the A-band. The thin filament is the regulatory structure, which consists of two strands of polymerized actin and two strands of tropomyosin. It also contains the troponin unit formed of three different subunits, the subunit C – Ca^{2+} binding component, I — inhibitory component, and T — tropomyosin-binding component, altogether representing the Ca^{2+} sensor. The myosin molecules in the sarcomere are linked together in their center at the level of M-line. Z-line joins the actin filaments and contains other proteins, like α-actinin or desmin. One sarcomere is limited by two Z-lines.

Contraction takes place by the sliding of thin filaments between thick filaments due to the cyclic attachment of myosin heads to actin, i.e. formation of actomyosin cross-bridges (Ventura-Clapier et al, 1994) with the consumption of chemical energy liberated by the ATP hydrolysis in the presence of Ca^{2+}. Troponin C moiety in the regulatory complex has the key role in setting the activation characteristics of the contractile proteins (Holroyde et al, 1980). Cross-bridges can be classified into two states, a weakly bound state, where myosin binds ATP and strongly bound state with ADP or without bound nucleotides (rigor bonds) (Ventura-Clapier, et al., 1994). The transition between these two states is coupled to the release of Pi from the complex and leads to the generation of the force (Hibberd et al, 1985).

During the actomyosin cycle, four major steps can be outlined (Ventura-Clapier et al, 1994). (i) At the low Ca^{2+} concentration prevailing in diastole (about 200 nM), ATP is bound to myosin and hydrolyzed while the products, ADP and Pi, are not dissociated. The interaction of actin and myosin is inhibited and cross-bridges are detached. (ii) After Ca^{2+} binding to TNC, thin filaments are activated and actomyosin complexes are formed, cross- bridges are attached. (iii) Release of the products of ATP hydrolysis takes place and leads to the formation of rigor bonds with conversion of chemical energy to mechanical work. (iv) Dissociation of actin and myosin after fixation of a new ATP molecule on myosin and relaxation of the muscle. If Ca^{2+} is still present, the new cycle will be initiated. ATP binding and hydrolysis are considered to be fast and non-limiting processes of the whole cycle (Goldman et al, 1984). The Km for MgATP is in the order of 10 μM in myofibrils in the presence of an ATP regenerating system. The predominant steady-state intermediate of relaxed muscle is M•ADP•Pi. The release of products is activated 100 times by the binding of actin to myosin (Eisenberg et al., 1968). Pi release is associated with the force generating step which will be dependent on the mechanical constraints applied to the muscle. ADP release appears to limit the rate of cross-bridge detachment and maximum shortening velocity (Siemankowski et al., 1985, Dantzig et al., 1991). Therefore, accumulation of MgADP in the intramyo-

fibrillar space fixes the cross-bridges in their rigor states and by inhibiting the contraction with Ki being about 200 μM both in MgATPase reaction and in sliding of fluorescent actin on myosin (Cook and Pate, 1985, Yamashita et al., 1994) contributes to muscle fatigue.

3.2. Utilization of ATP for ion transport

3.2.1. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)

The SERCA transports Ca^{2+} from the cytosol into the lumen of SR at the expense of adenosine triphosphate (ATP) hydrolysis (Tada and Toyofuku, 2001). In contrast to F_0F_1-ATPase in the mitochondria it belongs to the group of P type ATPases, the catalytic cycle of which includes the intermediate phosporylation-dephosphorylation state of the enzyme with different conformations (Lauger, 1991). Molecular cloning analysis has identified three SR Ca^{2+}-ATPase genes, SERCA1, 2 and 3, which are spliced alternatively in several isoforms. SERCA1a is mainly expressed in fast-twitch skeletal muscle, while SERCA1b is abundant in fetal and neonatal stages (Brandl et al., 1987). Four splice variants encode for the SERCA2 isoforms, variant 1 translates in SERCA2a, which is the primary isoform expressed in cardiac and slow-twitch skeletal muscle tissue and variants 2–4 encode for SERCA2b, which is the predominant isoform in nonmuscle (variants 2 and 3) and neuronal cells (variant 4) (Wuytack et al., 1998). SERCA3 isoform is restricted to epithelial and endothelial cell types (Burk et al., 1989). The cardiac isoform of SERCA (SERCA2a) plays a pivotal role in the control of cardiac function: it is the principle means by which cytoplasmic [Ca^{2+}] is lowered during relaxation and also is the principle determinate of the concentration of Ca^{2+} in the SR, which, in turn, is an essential element in determining contractility due to its influence on the magnitude of Ca^{2+} release. SERCA2a activity is regulated by phospholamban (PLN) and its homologue, sarcolipin (SLN), through direct protein-protein interactions. A nonphosphorylated PLN inhibits the SERCA activity whereas phosphorylation of PLN relieves the inhibition (Koss et al., 1996, Brittsan et al., 2000, Kadambi et al., 1996). Thus, any alterations in the levels of PLN expression and/or phosphorylation have to be examined in parallel with possible alterations in the expression levels of SERCA2a. It is known that thyroid hormones are potent regulators of the PLN/SERCA2a ratio (Kaasik et al., 1997). In human heart failure, several lines of evidence suggest that there are alterations in intracellular Ca^{2+}-homeostasis, which may be related to an altered expression, function or regulation of Ca^{2+}-handling proteins. Along these lines, diminished peak and prolonged decay of Ca^{2+} transients have been reported by several groups in cardiomyocytes and papillary muscle strips isolated from hearts of patients with end-stage heart failure (Beuckelmann et al., 1992, Dipla et al., 1999). These alterations in the rise and fall of the Ca^{2+}-

transient have important implications for excitation-contraction coupling and the development of increased diastolic tension in heart failure. Furthermore, these alterations in Ca^{2+}-handling diminish frequency-potentiation, which is one of the potent intrinsic mechanisms inducing cardiac inotropy (Hasenfuss et al., 1994, Shwinger et al., 1995, Frank et al., 1998). Along them, it remains controversial whether SERCA2a expression changes in human heart failure. Several studies reported a downregulation of SERCA2a protein (Hasenfuss et al., 1994, Meyer et al., 1995), while others did not observe any significant changes of SERCA2a expression in end-stage failing human myocardium (Shwinger et al., 1995, Frank et al., 1998). A decrease in the expression of SERCA2a may account for increased PLN to SERCA2a ratios and thus for increased inhibition of the affinity of SERCA2a and prolonged relaxation (Shwinger et al., 1995).

3.2.2. Na^+-K^+-ATPase

The Na^+-K^+-ATPase is another member of the P-type ATPases. The Na^+-K^+-ATPase has two major subunits: α and β (Blanco et al., 1998). The α subunit has a molecular weight of ~110 kDa and contains the binding sites for ATP, Na^+, K^+ and cardiac glycosides (specific inhibitors of the enzyme). The smaller β subunit (~50 kDa) modulates the ATPase activity and is important in the proper membrane insertion of the pump. A third, smaller (~12 kDa) protein (γ subunit) has also been found in various tissues (Mercer et al., 1993) but its physiological function is not yet known. Four α (α_1–α_4) and three β (β_1–β_3) subunits of the Na^+-K^+-ATPase have been identified. The α_1–α_3 isoforms are expressed in a variety of tissues, whereas the α_4 isoform has only been detected in rat testis (Shamraj et al., 1994). Any combination results in a functional pump. All three α-isoforms are present in the human heart (Zahler et al., 1994, Wang et al., 1996, McDonough et al., 1996), The β_1 isoform is the only β subunit appreciably expressed in the human heart, although a recent report indicated that β_3 is also present (Pierre et al., 2001).

Different Na^+-K^+-ATPase isoforms have different sensitivities towards cardiac glycosides and $[Na^+]_i$. In human, the α_1 isoform is much more sensitive to ouabain, (Wang et al., 2001) than in rat. Expression of Na^+-K^+-ATPase is higher in ventricle than atrium (Wang et al., 1996). In ventricular myocytes, Na^+-K^+-pumps are located in both peripheral sarcolemma and T-tubules (McDonough et al., 1996). In the rat, the α_1 isoform is preferentially distributed in T-tubules, whereas α_2 and β_1 are homogeneously distributed in the T-tubules and peripheral sarcolemma (McDonough et al., 1996). The level of expression of different isoforms and/or their cellular localization could have important physiological consequences.

The Na^+-K^+-ATPase transports three Na^+ ions out and two K^+ ions into the cell using the energy of one ATP molecule, and thus moves out one net charge

per cycle. The $[Na^+]_i$ for half-maximal pump activation (K_m) in the heart varies widely with the internal and external ionic conditions, in the range of 8–22 mM (Glitsch, 2001). Intracellular K^+ competes with Na^+ for binding to the enzyme at the cytoplasmic surface and results in reduced $[Na^+]_i$-sensitivity of the pump (Therien and Blostein, 1999). The activating *Km* for extracellular K^+, in the presence of normal external $[Na^+]$, is 1–2 mM (Glitsch, 2001), therefore the pump is ~70% saturated with respect to external K^+ at a normal concentration of 4 mM. External Na^+ and K^+ compete for common binding sites to the Na^+-K^+-ATPase, therefore the *Km* for pump activation by external K^+ is appreciably lower in Na^+-free conditions. The ATP concentration for half-maximal activation of the cardiac Na^+-K^+-pump is in the range of 80–150 μM (Hilgemann et al., 1991, Friedrich et al., 1996), therefore under control conditions ATP is not rate limiting for the pump (normal ATP levels in cardiac cells are 5–10 mM). However, this can change as local [ATP] declines during ischemia or metabolic inhibition, and the simultaneous rise in [ADP] and $[P_i]$ contribute to a reduced ΔG_{ATP} available for transport (Nicholls and Ferguson, 2002). Such a reduction in ΔG_{ATP} would also reduce the $[Na^+]$ and $[K^+]$ gradients that the pump can generate. However, during short-term metabolic inhibition glycolysis may regenerate ATP near the Na^+-K^+-ATPase, making the pump less directly dependent on oxidative phosphorylation (Glitsch et al., 1993).

4. The problem of regulation of the energy metabolism *in vivo*

4.1. Regulation of mitochondrial respiration in vitro versus in vivo

The mechanisms of the regulation of mitochondrial respiration, ATPases and intracellular energy transfer described above have been revealed mostly in the experiments where the isolated cellular structures (mitochondria, sarcolemma, SR, and purified enzymes) have been studied. Along with these studies the parallel registration of cardiac work and energy metabolism in intact cardiac preparations (e.g. Langendorff heart) or working heart by Neely et al. (1972), i.e. in conditions *in vivo* have revealed the challenging paradox (Neely et al., 1972, Williamson et al., 1976, Balaban et al., 1986, Wan et al., 1993, revised by Saks et al, 2004). It was shown that increasing in cardiac workload either by stretching the muscle length (Frank-Starling mechanism) or by enhancing the pumping frequency linearly enhances the tissue respiration without significant fluctuations in intracellular PCr, Cr, ATP and ADP levels, this termed as a status of metabolic stability (Neely et al., 1972). This is not the case for glycolytic skeletal muscles, however, in which increasing workload is associated with significant increase in ADP and Pi levels together with decreased PCr and ATP levels (Kushmerick et al., 1992). The metabolic stability in intact oxidative

muscle cells means that the cytoplasmic ADP levels are dissociated from respiration rate and workload levels. To solve the conflict between these two physiological observations — linear dependence of the respiration rate upon the workload and metabolic stability of the heart — it was proposed that the respiration rate *in vivo* is not at all regulated by ADP, but by Ca^{2+} in parallel with regulation of contraction ("parallel activation theory") (Hansford, 1985, McCormac et al., 1990). Indeed, this idea is plausible, since both the mitochondrial dehydrogenases and actomyosin complexes (contraction) can be activated by Ca^{2+} synchronously (Hansford, 1998, Denton et al., 1972, 1978, McCormac et al., 1990). The principal question here is whether the real changes in intracellular [Ca^{2+}] transients can explain the 15–20-fold changes in the respiration rate under conditions of Frank-Starling law and metabolic stability *in vivo*? In fact, the answer to that question was found to be "No" throughout the following experiments. (i) Monitoring of intracellular [Ca^{2+}] by special probes revealed that a stepwise stretch of the myocardium produced a rapid potentiation of twitch force but not the Ca^{2+} transient (Kentish and Wrzosek, 1998, Shimizu et al., 2002). (ii) When the effects of Ca^{2+} on the respiration, F_oF_1 ATPase and $\Delta\Psi$ in isolated heart mitochondria were studied, it was found that changes in mitochondrial [Ca^{2+}] although being rapid enough to participate in regulation of respiration, can increase the respiration rate only up to 2 times with an increase of the free cytoplasmic [Ca^{2+}] up to 600 nM (Territo et al., 2000, 2001). (iii) It is known that physiologically the respiration rates can be elevated ten-fold and that the mean cytoplasmic [Ca^{2+}] may extend up to 1–3 μM (Hansford, 1985) which clearly exceeds the saturation level of the mitochondrial Ca^{2+}-sensitive enzymes. The conclusion from these studies is that mitochondrial respiration should always proceed at rates closer to Vmax. Hence, the theory of parallel activation fails to explain the main physiological phenomenon, the 15–20 fold changes in respiration rate in cardiac cell induced by Frank-Starling mechanism under conditions of metabolic stability *in vivo*. However, it is likely that increased cytoplasmic Ca^{2+} keeps the mitochondrial systems in an activated state, being ready for regulation by metabolic signals.

Among the variety of metabolic signals, ADP plays a predominant role in stimulating the mitochondrial respiration since increasing of [ADP] near ANT activates the respiration in accordance to Michaelis-Menten relationship, with apparent affinity of ≈10 μM (Chance and Williams, 1956). If one considers the mitochondrial activation *in vivo*, the principal question is how can mitochondrial oxidative phosphorylation be activated in conditions of metabolic stability, when the cytoplasmic ADP levels practically do not increase despite maximal contractile activation. The studies started in seventies of last century have revealed that effective control over local ADP concentration near ANT can be achieved by functional coupling of creatine kinases.

4.2. The coupled creatine kinase reactions

4.2.1. Mitochondrial creatine kinase

Since the pioneering studies by Belitser and Tsybakova (1939) on muscle homogenates showing the activation of respiration by creatine at constant PCr/O_2 ratio, it became appreciated by early seventies that the mitochondria isolated from muscles effectively synthesize PCr in conditions of oxidative posphorylation and in the presence of creatine (Bessman and Fonyo, 1966, Vial et al., 1972, Jacobus and Lehninger, 1973, Saks et al., 1974, 1975). These experiments together with discovery of mi-CK and its structural localization in the outer aspect of the inner mitochondrial membrane (Scholte et al., 1973) and formation of mi-CK complexes between inner and outer mitochondrial membranes (Schlegel et al., 1988, Schnyder et al., 1994, Stachowiak et al., 1998) gave rise to the concept of functional coupling between mi-CK and ANT (Saks et al., 1994). Accordingly, it is assumed that ATP generated by the mitochondria and exported to the intermembrane space by ANT is converted into PCr by mi-CK. Another product of the CK reaction ADP is returned into the matrix by ANT (Saks et al., 1994, 2004, Joubert et al., 2002). Due to such a tight interaction between mi-CK and ANT the oxidative phosphorylation itself controls the PCr production in heart mitochondria, by shifting the mi-CK reaction out of the equilibrium — towards PCr synthesis. On the other hand, mi-CK stimulates oxidative phosphorylation by providing ADP for translocation into matrix and removing ATP from its binding sites with ANT.

Experimentally, the role of functional coupling between mi-CK and ANT was verified recently in the studies of the energy metabolism in the heart of mice with knock-out of mi-CK: as predicted by the theory described above, these hearts had lower levels of the phosphocreatine and reduced post-ischemic recovery (Spindler et al., 2002, 2004).

4.2.2. Myofibrillar creatine kinases

The myofibrils isolated and purified from muscle cells contain large activities of MM-CK (Saks et al., 1977), localized mostly in M-line of the sarcomere (Wallimann et al., 1984, Hornemann et al., 2000) and in I — band of sarcomeres (Wegmann et al., 1992). There is an increasing amount of evidence that this MM-CK is intimately involved in the contraction cycle at the level of the ADP release and ATP rebinding steps. (i) It has been shown that PCr accelerates the release of muscle from rigor tension in the presence of exogenous ATP, decreasing the necessary relaxing ATP concentration by order of magnitude (Ventura-Clapier et al., 1987, Ventura-Clapier et al., 1998). (ii) Krause and Jacobus (1992) have shown close functional coupling between the actomyosin ATPase and the CK reaction in isolated rat heart myofibrils, seen as the decrease of the apparent Km

value for ATP in ATPase reaction. (iii) Sata et al. (1996) found that sliding velocity of fluorecently labeled actin on an immobilized cardiac myosin showed significantly smaller apparent Km for MgATP than in the absence of CK. Ogut and Brozovich (2003) studied the kinetics of force development in skinned trabeculae from mice hearts and found that in spite of the presence of 5 mM MgATP, the rate of force development depended on the concentration of the PCr, and concluded that there is a direct functional link between the CK reaction and the actomyosin contraction cycle at the step of the ADP release in myofibrils. Most probably, this effective interaction occurs in small microcompartments in myofibrils where the local adenine nucleotide pool can be shared between MM-CK and ATPase. Both, the mathematical modelling of the myofibrillar CK reaction (Aliev and Saks, 1997, Saks and Aliev, 1996) and ^{31}P-NMR inversion transfer studies (Joubert et al., 2004) showed that myofibrillar CK is out of equilibrium following the contraction cycle. Thus, the role of MM-CK in myofibrils may be envisaged as follows. An increase of the number of active cross-bridges due to the Frank-Starling phenomenon during workload changes results in the rapid utilization of MgATP by cross-bridge cycling. The product of actomyosin ATPase – ADP is rapidly removed by MM-CK that also replenishes MgATP for myosin ATPase reaction. Thus, interaction between MM-CK and myosin ATPase allows to avoid inhibition of cross-bridge cycling by accumulation of MgADP near the ATPases. On the other hand, high local value of the MgATP/MgADP ratio and thus the local phosphorylation potential (high ΔG for ATP hydrolysis) can be maintained.

4.2.3. Membrane-bound creatine kinases

MM-CK is bound not only to myofibrils, but also to SR and sarcolemma. Numerous studies have revealed that MM-CK in these structures is also coupled to corresponding transport ATPases (Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase, respectively) (Rossi et al., 1990, Minajeva et al., 1996, Korge et al., 1993, 1994). Like in myofibrils, the MM-CK prevents accumulation of ADP in microdomains close to the ion pumps, thereby avoiding inhibiton of their function.

4.2.4. Concept of intracellular energy transfer

Discoveries of functionally coupled systems of creatine kinases resulted in a concept that mitochondria and ATPases interact via CK energy transfer network. Accordingly, due to functional coupling between mi-CK and ANT ATP generated by the mitochondria and exported to the intermembrane space by ANT is converted into PCr by mi-CK, and PCr is used to locally rephosphorylate ADP formed by ATPases (Saks et al., 1994, Joubert et al., 2004). These sites are connected through the near-equilibrium CK reactions

with participation of cytosolic MM-CK (Dzeja and Terzic, 2003) that guarantee very fast transfer of energy rich phosphoryl groups without significant fluctuations of cytosolic adenine nucleotide concentrations. High [ATP]/[ADP] ratio near the ATPases sustained by this system ensures the maximal free energy liberated from splitting of γ-phosphate group of ATP (Nicholls and Ferguson, 2002. It is generally accepted that in normal heart, the CK phospho-transfer system represents the predominate way of energy transduction (Dzeja et al., 2003, Saks et al., 2004). However, besides this system there exists also the AK-phosphotransfer system, which operates due to coupling of mi-AK to ANT in mitochondria and interaction of AK2 isoform with ATPases (Dzeja and Terzic, 2003; Dzeja et al., 1985). It has been shown that ADP produced locally by mitochondrial kinases is more effective in stimulating mitochondrial respiration than bulk ADP. These systems together allow effectively to match the increased energy demand with enhanced energy production in response to increased workload.

5. Energy metabolism in fibrillating atria

Atrial fibrillation is the most frequent dysrhythmia in humans, with congestive heart failure for being the strongest clinical predictor (Benjamin et al., 1994). It is associated with structural remodeling characterized by partial myolysis, interstitial fibrosis, deposition of fat and glycogen, enlargement of atria and impaired atrial contractility. To date the mechanisms of atrial fibrillation have been largely ascribed to electrical remodelling, characterized by altered Na^+ and Ca^{2+} cycling between extra- and intracellular compartments and Ca^{2+} overload associated with electrical remodeling, expressed by shortened AP duration and partial depolarization of resting membrane potential, these changes leading to shortened effective refractory period as a major basis for arrhythmias (Van Wagoner and Nerbonne, 2000, Schotten et al., 2002). Studies on the Ca^{2+} handling in fibrillating atria have revealed decreases in the L-Ca^{2+} channel density together with $I_{Ca2+,L}$ current, transient outward K^+ current (I_{TO}), ultrarapid delayed rectifier K^+ current (I_{Kur}) (Van Wagoner and Nerbonne, 2000), but increases in Na^+/Ca^{2+} exhanger (Schotten et al., 2002) and Na^+-K^+-ATPase (Maixent et al., 2002), without any change in SR Ca^{2+} pump protein density in human fibrillating atrial myocytes. These observations are paradoxical, since neither of them can cause Ca^{2+} overload; on the contrary, they should either attenuate or avoid accumulation of excess Ca^{2+} in the cells. When one seeks for other potential reasons for development of arrhythmias, a wealth of evidence pointing to possible role of alterations in the energy metabolism cannot be overlooked. Both of the studies of atrial fibrillation in animal models of humans have revealed the decreased tissue levels of ATP and PCr (Cha et al., 2003). Studies on dog model of AF have shown that the atria with more

expressed decrease in tissue ATP concentration are more prone to sustained AF as well (Cha et al., 2003). It follows from these data, that all the factors capable of attenuating the intracellular ATP levels can be viewed as potential factors causing the AF. The recent studies have shown that atrial fibrillation in humans is associated with accumulation of 4977 bp (Lai et al., 2003, Lin et al., 2003) or 7436 bp mtDNA (Tsuboi et al., 2001) deletions, occurring independently of age. Since the 4977 bp deletions are considered to affect synthesis of all 13 mitochondrially encoded proteins, the components of complex I, III, IV, and ATP synthase (Lai et al., 2003, Lin et al., 2003), one may expect the processes of oxidative phosphorylation to be disturbed, that may be responsible for decreased ATP levels. On the other hand, the mtDNA deletions have been found to be associated with upregulated AMP deaminase activities and decreased total amount of adenine nucleotides (ATP, ADP and AMP) (Tsuboi et al., 2001, Tomikura et al., 2003). Thus, ATP synthesis may be attenuated not only due to defective respiratory chain but also due to rapid degradation of its precursors. However, the oxidative phosphorylation has not been studied in fibrillating atria. Besides these mechanisms, reduced activities of MM-CK owing to protein oxidation in myofibrils of fibrillating human atria (Mihm et al., 2001) may represent a critical factor for decreased [ATP] — if rephosphorylation of ADP produced by ATPases at the expense of PCr can not be effective, the tissue ATP levels must fall. Studies on rat atria have shown that mi-CK is not coupled to oxidative phosphorylation in this species (Anflous et al., 1997). This observation points to a possibility that CK-energy transfer is not operable in atria, this would limit the atrial capacity to synthesize PCr, and thereby sustain rephosphorylation of ATP for ATPases. Deficit in cellular AMP levels associated with that process may limit ATP supply for ATP-dependent K^+ channels by AK-phosphotransfer network (Selivanov et al., 2004) Thereby decreasing atrial refractoriness, a major condition for atrial arrhythmia (Van Wagoner and Nerbonne, 2000).

At present it is not clear what causes remodeling in structural, electrical and energetic remodeling in fibrillating atria. One potent factor that should be taken into account is atrial stretch due to volume overload. The shift from α- to β-myosin isoenzyme distribution has been shown to occur in myosin human atria as an early response to a hemodynamic load, so that only slightly enlarged atria tend to have higher β-isoenzyme (Buttrick et al., 1986). Patients with larger atria have more pronounced atrial stunning after cardioversion of the atrial fibrillation and they recover from stunning later than patients with normal size of the atria (Khan, 2002). Passive atrial stretch increases the oxygen consumption (Bittl and Ingwall, 1986), which together with reduced atrial flow (Jayachandran et al., 1998) and increased metabolic demand (White et al., 1982) may result in ischemia. As a result the reactive oxygen species may accumulate in mitochondria, causing oxidative damage of the respiratory chain proteins and CK isoenzymes and creatine kinases (Mekhfi et al., 1996, Mihm et al., 2001). In hypoxic/ischemic conditions the ATP synthase may reverse to consume ATP,

which may be augmented due to increased expression of ATP-hydrolysing subunit of the ATP synthase complex. The extra ADP produced by F_0F_1-ATPase would be transferred to ATP-dependent K^+ channels, causing their opening (Carrasco et al., 2001), as one of the potential reasons for fibrillation. The atrial stretch can also directly increase the transsarcolemmal influx of Ca^{2+} ions (Calaghan and White, 1999, von Levinski et al., 2003). Excess intracellular Ca^{2+} in turn causes swelling of mitochondria, probably due to opening of the PTP. In addition the Ca^{2+}-dependent calpains are activated that results in proteolysis of contractile, L-type Ca^{2+} channel, and cytoskeletal proteins (Brundel et al., 2002). Altogether these changes result in structural remodeling of atrial myocardium, characterized by perinuclear loss of sarcomeres and SR, dedifferentiation of the cardiomyocytes (Rücker-Martin et al., 2002), cellular hypertrophy and increased fibrosis (Van Wagoner and Nerbonne, 2000). These processes may affect the cellular energy metabolism compartmentalized into ICEUs differently throughout the development of disease, depending on duration of the disease and the balance between the energy supply and demand. For example, Ausma at al. (2000) have found that after initial decrease in cellular PCr during 8 weeks content this parameter recovered by 16th week of experimental atrial fibrillation. It was suggested that this change reflected the development of new steady state, characterized by slow rate of energy consumption due to reduction of myocardial function.

6. Role of cytoskeleton in heart diseases and skeletal muscle dystrophies

The cytoskeleton is a well-organized structure representing a scaffold within the cells that maintains the various subcellular organelles in their normal spatial arrangement. It contributes to many of cellular functions such as preservation of cell shape and volume, mechanical resistance, contractility, intracellular signalling, and cell-to-cell interaction. On structural basis the cytoskeleton can be classified according to the diameter of the component fibers: microfilaments of actin (diameter 6 nm), intermediate filaments (10 nm) such as desmin and vimentin, and microtubules (25 nm) (Rappaport et al., 1998). Based on their function the cytoskeletal proteins can be divided into four different groups (Hein et al., 2000).

I. Sarcomeric skeleton: titin, C-protein, α-actinin, myomesin, and M-protein.
II. True cytoskeletal proteins: tubulin, desmin, and actin.
III. Membrane associated proteins: dystrophin, spectrin, talin, vinculin, plectin and ankyrin.
IV. Proteins of the intercalated disc: desmosomes consisting of desmoplakin, desmocollin, desmoglein and desmin; adherens junctions with N-cadherin, the catenins and vinculin and gap junctions with connexin.

The major component of the **microfilaments** is actin, which exists in 6 isoforms. 4 α-actins (skeletal, cardiac, vascular and enteric) are found in sarcomeric structures, while β- and γ-actins are predominantly cytoplasmic (Stromer 1998). Actin filaments consist of two-strand polymerized actin monomeres. The non-sarcomeric actin appears to be necessary for cell spreading and the generation of new myofibrils (Rothen-Rutishauser et al., 1998). Actin also participates in formation of connections with the extracellular matrix via membrane-spanning integrins at sites close to the Z-line known as costameres. Actin associates with integrins via actin binding proteins e.g. vinculin, α-actinin, talin and paxillin. Actin also connects to other membrane spanning proteins such as β-dystroglycan via dystrophin (Karelian and Severs, 2000). The non-sarcomeric actin microfilaments can be easily visualized by immunfluorescence in cultured neonatal cardiac cells, in accordance with their important role in cell spreading. However, in adult cardiomyocytes they can be sparsely seen, with increasing amounts in hypertrophied cardiomyocytes (Calaghan et al., 2004).

To assess the role of actin in the cardiac cells different agents modulating its structure have been applied. Among them cytochalasin-D, latrunculin A, DNase I, gelsolin and cofilin disrupt filamentous actin. Conversely, filamentous actin can be stabilised by phalloidin (Calaghan et al., 2004). Application of these pharmacological tools has revealed that nonsarcomeric actin cytoskeleton modulates the electrical activity and ion channels (L-Ca^{2+} channels, K^+-channels, stretch activated channels, chloride channels and Na^+-Ca^{2+}-exchanger in neonatal and cultured myocytes). In the adult myocytes the relevant role is not convincingly demonstrated partially because sarcomeric actin modulates the amount of intracellular Ca^{2+} available.

The **microtubules** are the major component of the cardiac cytoskeleton with many roles in protein synthesis, intracellular trafficking and signalling (Calaghan et al., 2004. Hein et al., 2000). The tubulin molecule is a heterodimer of an α- and β-isoform with a molecular weight of 55 kDa per monomer. By polymerizing these heterodimers form the hollow protein cilinders of 25 nm in diameter (Calaghan et al., 2004, Hein et al., 2000). The polymerized tubulin consist only 30% of its cellular pool, with the rest of 70% occuring in non-polymerized form. However, both polymerized and free tubulin heterodimers have been considered to participate in cell signalling. The microtubules can bind the GTP-binding proteins (Gi and Gs) which power their polymerization and microtubule associated proteins (MAPs) that promote microtubule stability, Tau protein, kinases and molecular motors (kinesin). The microtubules are associated with actin and intermediate filaments, and via MAPs they are able to connect with actin microfilaments and microtubules (Calaghan et al., 2004). The microtubules bind to mitochondria via MAP at the outer mitochondrial membrane, probably at the VDAC2. The microtubules participate in axonal mitochondrial transport in neurons with participation of kinesin and dynein.

To study the role of microtubules their structure can be altered by colchicine which causes disruption of the microtubules and taxol, which causes proliferation and stabilisation of the microtubules. A number of studies have revealed important role of microtubules in development of cardiac hypertrophy and failure. In feline model of cardiac hypertrophy the contractile dysfunction and loss of compliance was associated with increased cytoskeleton stiffness, augmentation of total amount of tubulin and elevated degree of its polymerization. Reduction of microtubule hyperpolymerization by colchicine reversed the myocyte stiffness and normalized the contractile parameters. Taxol treatment of normal myocytes causes accumulation of microtubules and functional disturbances similar to those *in vivo*. It has been concluded, therefore, that cytoskeletal abnormalities rather than changes in contractile apparatus are causal for contractile dysfunction observed in compensated hypertrophy and failing heart (Hein et al., 2000, Kostin et al., 2003). There is agreement that in failing heart the tubulin expression is clearly increased, together with vinculin, vimentin and fibronectin, and that these changes occur in parallel to increased left ventricular end diastolic pressure in humans (Heling et al., 2000).

Desmin (MW 53 kDa) belongs to the family of intermediate filaments by forming a dimer of two α-helical chains interwined in a coiled-coil rod. In striated muscle cells desmin forms a physical link between the nucleous, contractile proteins (by surrounding the Z-discs), sarcolemma and extracellular matrix via costameres by forming three-dimensional scaffold that surrounds the Z-discs, extending from one Z-disc to other, and seems to potentially associate with mitochondria and SR, as well as with T tubules (in case of cardiac cells). The desmin also extends from the Z- disc to the plasma membrane at the levels of costameres and intercalated discs, project from the Z-dics of the perinuclear myofibrils to the nuclear membrane, and form a longitudinal lattice around the myofibrils. The desmin filaments cannot be selectively manipulated with pharmacological tools. However, as reviewed by Capetanaki, 2002, their important physiological role has been convincingly demonstrated in desmin null mice. The lack of desmin results in hypertrophy of myocytes, increased thickness of free ventricular wall, further on a ventricular dilatation and decreased force and pressure development (depressed Frank-Starling mechanism), but without change in myofilament Ca^{2+} sensitivity. At ultrastructural levels the degenerated cardiomyocytes with disintegrated myofibrils are observed. The mitochondrial distribution is rearranged as they accumulate into subsarcolemmal clumps. The mitochondria extensively proliferate and associate with mitochondria and sarcoplasmic reticulum, probably via cytolinker proteins such as plectin. It appears therefore, that loss of desmin disintegrates the interconnection between the mitochondria and other cellular structures. Several data point to important functional consequences of that process, as mitochondria in skinned fibers exhibit increased apparent affinity to exogenously added ADP in regulation of respiration, decreased maximal rate of respiration and impaired functional coupling of mi-CK and ANT (Kay et al., 1997, Capetanaki, 2002). It has been

suggested that desmin can influence the mitochondrial function by altering the proportion of subsarcolemmal and interfibrillar mitochondria, by increasing the former fraction with lower content of respiratory chain compared to latter. Desmin may also regulate mitochondrial shape, by contracting and stretching the mitochondrial membrane, in association to contraction and relaxation cycle of the sarcomere. Its effect on mitochondrial affinity towards ADP can be modulated by formation or stabilization of the mitochondrial contact sites between inner and outer mitochondrial membranes, or by binding to VDAC directly or through MAP2 or plectin (Leterrier et al., 1994, Reipert et al., 1999)

There exist an increasing number of data showing that lack of desmin leads to cell death and heart failure. The desmin null mice exhibit dilatation of the ventricular chamber. It is likely that they are more prone to opening of the PTP as suggested by appearance of swollen mitochondria, that simulated the apoptotic cell death (Capetanaki, 2002). It has been shown that expression of desmin increases in a course of experimental cardiac hypertrophy, probaly to keep sarcomeres in a register. A progressive increase in desmin protein and filaments was observed during the transition from hypertrophy to failure in guinea pig hearts and in explanted failing human myocardium (Hein et al., 2000). Li et al (1999) have show that a novel missense mutation of desmin, Ile451Met, is responsible for idiopathic dilated cardiomyopathy in family 220-032.

Dystrophin is a 427-kDa protein with binding affinity to cytoskeletal actin and to transmembrane protein β-dystroglycan, a surface membrane receptor for the extracellular matrix component laminin, suggesting that dystrophin provides a structural link between the myocyte cytoskeleton and extracellular matrix. Genetic mutation in dystrophin (X-linked trait) is the most common cause of muscular dystrophy accounting for both the Duchenne and Becker phenotypes of disease (Kaprelian et al., 2000, Watchko et al., 2002). While DMD is characterized by an absence of stable dystrophin molecules, this protein is synthesized in less than normal amounts in Becker muscle disease (BMD). Correspondingly, the symptoms of the disease — progressive muscular weakness and atrophy are less expressed in BMD. To understand the mechanisms of DMD or BMD, the muscles of the dystrophin knockout mice (MDX mice) have been extensively studied. The results demonstrate altered CK system, as expression of the mi-CK gene is decreased in the hindlimb muscles and diafragm of the 3-month-old MDX mouse (Tkatchenko et al., 2000). Similarly to a mice model, the impaired CK has been revealed in human muscles, for the patients with DMD or BMD display decreased contents of PCr but increased ADP (assessed by ^{31}P-NMR spectroscopy) in leg muscles, these changes increasing after exercise and being more prominently expressed in patients with DMD than with BMD (Dupont-Versteegden et al., 1994, Kemp et al., 1993). The lack of dystrophin appears to influence the oxidative phosphorylation, based on disturbances observed in mitochondria isolated from the MDX muscles (Glesby et al., 1988, Dunn et al., 1993, Even et al., 1994, Dupont-Versteegden et al., 1994). However these data are still inconsistent, since the ^{31}P-NMR studies in patients (Kemp et al., 1993,

Lodi et al., 1999) and experiments on skinned cardiac fibers from MDX mice show either a normal capacity of mitochondria to produce ATP, or the muscle type-dependent alterations in oxidative phosphorylation (Kuznetsov et al., 1998). The controversies can be attributed to different character and time scale of the balance between dystrophic and regenerative processes in humans and mice. Indeed, in contrast to humans who die due to DMD in their childhood, the MDX mice exhibit practically normal life span (Deconinck et al., 1997). It has been revealed in MDX mice that the myopathic lesions progress to a peak of degeneration characterized by extensive mitochondrial deterioration at 5–6 weeks (Dupont-Versteegden et al., 1994, Cooper, 1989, Dunn and Radda, 1991, Turner et al., 1988, Lucas-Heron, 1996, De la Porte et al., 1999). Then a regeneration period follows when a number of processes being controlled by the genetic programme of the fetal myogenesis occur (Helliwell et al., 1992, Megeney et al., 1996). The regeneration processes are completed at the age of 12–16 weeks (Cooper, 1989). Among those the expression of utrophin is important, as by substituting dystrophin it prevents progression of necrotic dystrophy and premature death (Helliwell et al., 1992, Sweeney, 1993, Pasternak et al., 1995). Based on observation that the skeletal muscles of MDX mice exhibit 58 genes differentially expressed compared to wild-type mice (Boer et al., 2002), the compensatory processes seem to be much more complex. The genes involved belong to those responsible for control over muscle development genes, immune response, proteolysis and extracellular matrix modelling. According to the recent data, lack of dystrophin attenuates or stimulates the expression of number of the proteins, such as titin, troponin I, α-tubulin and Rac1 which participate in structural organization of the muscle cells (Tkatchenko et al., 2000). In addition, it is known that the cytoskeletal proteins can modulate the function of the contractile proteins (Stromer, 1998), that about 40% of dystrophin is tightly bound to the contractile apparatus, and that a loss of that dystrophin fraction is critical for the development of cardiac insufficiency (Meng et al., 1996). Interestingly, some of genes related to energy metabolism, such as AMP deaminase, lysosomal acid lipase, and creatine biosynthesis (glycine aminotransferase and guanidinoacetate *N*-methyltransferase) are upregulated (Boer et al., 2002). It is known that creatine, which increases muscle and brain PCr concentrations and protects mitochondria from PTP opening (Dolder et al., 2003) also protects from neuronal degeneration and improves restitution of muscles after heavy exercise (Wyss and Kaddurah-Daouk, 2000). Thus, increased capacity of biosynthesis of creatine may significantly contribute to faster regeneration of muscle cells in MDX mice and contribute to their long life span. At present, the function of CK system in conjunction with oxidative phosphorylation has been not studied in MDX mice. It has been shown that the skeletal muscle cells of MDX mice exhibit shift of the resting membrane potential towards more positive values (Duncan, 1989). Given that creatine transport depends on transmembrane Na^+ gradients (Wyss and Kaddurah-Daouk, 2000), this change may inhibit the creatine transport in the muscle cells of these mice.

AIMS OF THE STUDY

The general aim of the study was to investigate the energy metabolism in oxidative muscle cells *in vivo*, i.e. in natural intracellular interaction of mitochondria and ATPases and cytoskeletal structures. More specific goals were as follows:

1. To characterize the CK- and AK-mediated phosphotransfer systems in human atria.
2. To compare the basic parameters of oxidative phosphorylation in human atrial and ventricular myocardium.
3. To demonstrate that the CK- and AK-phosphotransfer networks and direct channeling of adenine nucleotides are compartmentalized into the ICEUs.
4. To characterize alterations in energy metabolism in human atria in conditions of atrial fibrillation.
5. To address the potential role of dystrophin in regulation of the mitochondrial function *in vivo* by using skinned muscle fibers of the MDX mice.
6. To compare two possible mechanisms (the uniform diffusion restriction and the localization of diffusion restrictions close to mitochondria) by which functional coupling between Ca,MgATPases and mitochondria could be formed in cardiac skinned fibers.

METHODS

1. Animals

The 10–11-month-old dystrophin knock-out homozygous female mice, provided by Prof. H. Jockusch's group (University of Bielefeld, Germany) and adult outbred Wistar rats of either sex weighing 250–350 g were used in the experiments. The animals were kept, fed and studied in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85 – 23, revised 1996).

2. Patients

The small specimens of atrial myocardium were obtained from two groups of the patients undergoing coronary artery bypass graft surgery, mitral or aortic valve replacement/correction and correction of atrial septal defect (ASD) at the Department of Cardiovascular and Thoracic Surgery of the University of Tartu. The sinus rhythm group (SR-group) (n=44), 25 males and 19 females, comprised the patients with coronary artery disease (CAD), aortic stenosis, and valvular diseases, but having a normal sinus rhythm. The patients of the "fibrillation" group (F-group) (n=15), 8 males and 7 females, had mostly the valvular diseases associated with the persistent AF. The transthoracical 2-D echocardiography in apical 4-chamber view was used to register the atrial geometry (maximal superior-inferior distance, S-ID), left ventricular ejection fraction (LVEF), valvular insufficiency, and pulmonary hypertension according to Weyman, 1994.

Compared to SR-group, the F-group patients exhibited enlarged right atrial chamber (S-ID of 4.2±0.1 cm, n=24 and 5.5±0.3 cm, n=12, respectively, $p<0.001$) associated with more frequent and severe tricuspidal insufficiency and pulmonary hypertension, a characteristic features of longstanding AF (Aimé-Sempé et al., 1999; Pozzolli et al., 1998). Before surgery the patients in F-group received digitalis glycosides complemented with Ca^{2+}-antagonists to control their ventricular rate and diuretics to reduce atrial overload. Part of the SR-group patients (CAD subgroup) was treated with nitrates and β-blockers, whereas the patients with valvular disease received ACE inhibitors and diuretics complemented, if necessary, with β-blockers and digitalis glycosides. As an outcome, the mean values of LVEF remained within the normal range (60±10%, [Weyman, 1994]), similarly in SR- and F-groups (57.1±1.6, n=42 and 55.9±2.7, n=14, respectively) and only few patients exhibited LVEF less than 50%. The SR- and F-groups did not differ by age (61.6±1.7, n=44, and 60.7±2.4, n=15, years, respectively) and gender.

The endomyocardial biopsies were taken from 16 patients (10 males and 6 females) suspected to suffer from dilatative cardiomyopathy at the University Hospital of Halle. Part of the muscle specimens was frozen in liquid nitrogen and stored at –70ºC for enzyme analysis, whereas another part was permeabilized (skinned) by saponin as described earlier (Seppet et al., 2001; Saks et al., 2001; Braun et al., 2001) and used for oxygraphical and morphological studies. The investigation is in conformity with the principles outlined in the Declaration of Helsinki (1997) and was undertaken with written consent from the patients and agreement of the local ethical commitee.

3. Imaging of mitochondria in skinned cardiac fibers

3.1. Laser confocal microscopy

The cardiac fibers were gently stirred in solution A in the presence of the mitochondrion-selective dye MitoTracker Green (Molecular Probes, Inc., Oregon, USA) (400 nM) in the dark for 30 min. Thereafter the fibers were washed 3 times in abovementioned solution without dye for 15 min by stirring, to minimize background fluorescence, placed on the specimen glass and covered by the cover glass without pressing them between the glasses. Some preparations were fixed by HistoPrep 10% prefilled buffered 10% formalin solution (Fisher Scientific, Pittsburgh, PA, USA) before microscopy. All procedures were performed at 20°C. The mitochondria were visualized and scanned by MRC 1024 BioRad (USA) laser confocal microscope. The specimen was illuminated by krypton/argon laser (15 mW) light (488 nm) and the emitted light signal was detected at 522 nm (DF35) and collected according to Kalman method using the BioRad aquisition system.

3.2. Transmission electron microscopy (TEM)

The myocardial specimens were fixed in 0.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in the same buffer. After dehydration with ethanol and acetone, the specimens were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in Tecnai 10 Electron Microscope (Philips, FEI Company, Netherlands) at 100 kV.

4. Determination of the tissue content of cytochrome aa_3

The tissue content of cytochrome aa_3 was assayed by registering the different spectra of reduced and oxidized cytochromes in cardiac homogenates according to Fuller et al., 1985 using Perkin-Elmer Lambda 900 spectrophotometer.

5. Analysis of the system of oxidative phosphorylation and coupling of oxidative phosphorylation to mi-CK and mi-AK and of the intactness of mitochondrial membranes

The function of the respiratory chain in skinned atrial fibers was assessed by using the oxygraphs (Oroboros, Paar KG, Austria or Rank Brothers Ltd, England) (Braun et al., 2001, Seppet et al., 2001, Saks et al., 2001) for measuring the rates of oxygen uptake (V_{O2}) in solution B.

The substrate specificity and function of the respiratory chain was polarographically (Oroboros, Paar KG, Austria or Rank Brothers Ltd, England) assessed as the respiration rates (V or V_{O2}) in condition of subsequent addition of 2 mM ADP to register the NADH-linked ADP-dependent respiration rate (V_{Glut}), 10 μM rotenone to inhibit the complex I, 10 mM succinate to activate FADH- linked ADP-dependent respiration (V_{Succ}), 0.1 mM atractyloside to monitor the respiratory control by adenine nucleotide translocase (ANT), 10 μM antimycin A to inhibit the electron flow from complex II to cytochrome c, 0.5 mM TMPD and 2 mM ascorbate to activate cytochrome oxidase (COX), and 8 μM cytochrome c to test the potential limitation of the respiration ($V_{Cyt\text{-}c}$) due to partial loss of cytochrome c, if the mitochondrial outer membrane (MOM) would have been impaired to the fibers incubated in solution B supplemented with 10 mM glutamate and 2 mM malate at 25ºC. The antimycin-sensitive respiration in the presence of atractyloside was considered to measure the excess respiration compensating the back flow of protons into the matrix (proton leak). In order to quantify the COX activity, the TMPD-dependent V_{O2} was inhibited by NaN_3 and the nonspecific V_{O2} was eliminated by plotting the TMPD-dependent V_{O2} against muscle concentration in the absence and presence of NaN_3.

The coupling between oxidative phosphorylation and mi-CK was estimated from two types of experiments. In one set, it was found from V_{O2} vs [ADP] relationships in the presence and absence of 20 mM creatine; the corresponding Km and Vmax values were calculated and the interaction between mi-CK and adenine nucleotide translocase (ANT) was expressed as [Km^{ADP}(-Cr)/ Km^{ADP}(+Cr)] (creatine index). In another protocol, 50 μM ATP was added after registration of the basal respiration rate (V_0) to achieve the limited stimulation

of mitochondria with endogenous ADP. Then the coupled reaction between mitochondrial AK (mi-AK) and ANT was activated by 2 mM of AMP, followed by quenching of the reaction with 0.2 mM diadenosine pentaphosphate (AP_5A). Coupling of mi-AK to respiration was quantified as the AK index (I_{AK}) calculated from equation $I_{AK} = [(V_{AMP} - V_{ATP})/V_{ATP}]$, where V_{AMP} and V_{ATP} are the respiration rates with AMP and ATP, respectively. After complete inhibition of AK 20 mM creatine was added to couple the mitochondrial CK (mi-CK) to ANT. The efficiency of coupling was expressed as the CK index (I_{CK}): $I_{CK} = [(V_{Cr} - V_{AP5A})/V_{AP5A}]$. Thereafter 2 mM ADP was added to maximally stimulate the oxidative phosphorylation (V_{ADP}). In the same protocol, the intactness of the mitochondrial inner and outer membrane (MOM) was also controlled by addition of 0.1 mM atractyloside and excess cytochrome c (8 μM), respectively (Saks et al., 1998). The maximal capacity of the respiratory chain was estimated as the V_{O2} with 2 μM FCCP (V_{FCCP}).

6. Determination of the competition between mitochondria and PK+PEP system and between MM-CK and PK+PEP system for ADP produced in ATPase reactions

6.1. Oxygraphic approach

Approximately 3–4 mg of fibers were incubated in solution B (1.5 ml) at 25ºC in the presence of 10 mM glutamate, 2 mM malate and 5 mM PEP. Coupling of mi-CK to oxidative phosphorylation was assessed by subsequent additions of 50 μM or 2 mM ATP, 20 IU/ml PK, 20 mM Cr, and 2 mM ATP. The same protocol was applied to estimate coupling between mi-AK and oxidative phosphorylation, except that 20 mM creatine was replaced by 2 mM AMP. The experiments were finished by addition of 0.1 mM ATR to monitor the respiratory control by endogenously produced ADP and intactness of the inner mitochondrial membrane (Saks et al., 1998).

6.2. Spectrophotometric approach

The fibers were incubated in the spectrophotometric (Perkin-Elmer Lambda 900) cuvette (1.5 ml) containing solution B complemented with 5 mM PEP, 0.24 mM NADH, PK (20 IU/ml) and lactate dehydrogenase (LDH) (20 IU/ml) and the rate of phosphorylation of ADP in PK reaction and direct transfer of ADP from ATPases to mitochondria were registered as previously (Braun et al., 2001, Seppet et al., 2001). The coupling of MM-CK to MgATPases was estimated as a decrease of the ADP flux through the PEP-PK system after addition of 20 mM phosphocreatine (PCr) in the presence of atractyloside.

7. Determination of the activities of AK and CK

The cardiac tissue frozen at –70°C was thawed at 0°C and homogenized in solution containing (in mM): EGTA 1, dithiothreitol (DTT) 1, $MgCl_2$, HEPES 5 and 1% Triton X-100 (1:20 w/v), pH 8.7 by an Ultra-Turrax homogenizer (Janke and Kunkel, Germany) (13500 rpm) on ice during 30 s followed by a 1 min period of standing on ice. This cycle of homogenization and standing was twice repeated and the homogenates were left on ice for 1 h for complete extraction of the enzymes. The AK activity was spectrophotometrically measured in the medium containing (in mM): Tris 20 (pH 8.0, 30°C), KCl 15, DTT 0.3, NADH 0.24, PEP 0.8, and 6 IU/ml PK and 3 IU/ml LDH. After registration of basal ATPase activity of the muscle homogenate in the presence of 1 mM ATP (together with 0.8 mM $MgCl_2$) 1.3 mM AMP was added to determine the AK activity from the changes in NADH oxidation rates at 340 nm. The reverse CK activity was measured in the medium containing (in mM): glucose 20, AMP 20, DTT 0.3, Mg-acetate 3, NADP 1, ADP 1, Tris-HCl 50 (pH 7.4, 25°C) in the presence of 2 IU/ml hexokinase and 2 IU/ml glucose-6-phosphate dehydrogenase, by monitoring the increase in NADPH formation at 340 nm after starting the reaction with 20 mM PCr. The CK isoenzyme profile was assayed electrophoretically (Vannier et al., 1995).

8. Mathematical modeling

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

9. Reagents and solutions

All reagents were purchased from Sigma (USA) except the enzymes which were obtained from Serva (Germany). Solution A contained, in mM:

CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol (DTT) 0.5, potassium 2-(N-morpholino)ethansulfonate (K-Mes) 50, imidazole 20, taurine 20, Na_2ATP 5.3, phosphocreatine 15, pH 7.1 adjusted at 25°C. Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3, and 5 mg/ml bovine serum albumine (BSA), pH 7.1 at 25°C. The stock solutions of ATP and ADP used to add ATP or ADP into solutions contained $MgCl_2$ (0.8 mol/mol ATP or 0.6 mol/mol ADP).

10. Statistical analysis

One way ANOVA with Newmann-Keuls or Bonferroni's multiple comparison test was used for data analysis. For comparison of two groups of data the Student's t-test was applied. The means $\pm$ SEM are presented if not otherwise stated.

RESULTS AND DISCUSSION

1. Energy metabolism in human heart (Papers II and IV)

1.1. Function of the respiratory chain and its coupling to phosphorylation

Fig. 1 shows that the atria exhibited very low basal respiration rate (V_0) in the presence of glutamate/malate. However, the addition of saturating concentrations of ADP increased it 6–7-fold (V_{Glut}) above the basal level. This ratio of V_{Glut} to V_0 indicates effective respiratory control by ADP in skinned fibers (Saks et al., 1998). In the presence of rotenone that fully inhibited the complex I, succinate reactivated the respiration (complex II-dependent state 3 respiration, V_{Succ}). Atractyloside suppressed the respiration by blocking ANT, but further inhibition was achieved with antimycin. The antimycin-sensitive portion of respiration was taken to measure the proton leak, whereas the ratio of respiration with succinate (V_{Succ}) to that with atractyloside (V_{Atr}) was considered to be the respiratory control index (RCI_{Succ}) for mitochondria with FAD-linked substrates. The COX activity was estimated as the NaN_3 dependent respiration rate (V_{COX}) in the presence of TMPD. Table 1 summarizing the results demonstrates that in SR-group the V_{COX} exceeded the V_{Succ}, but it was not different from V_{Glut}. The V_{Glut} was above the V_{Succ}, this resulting in succinate

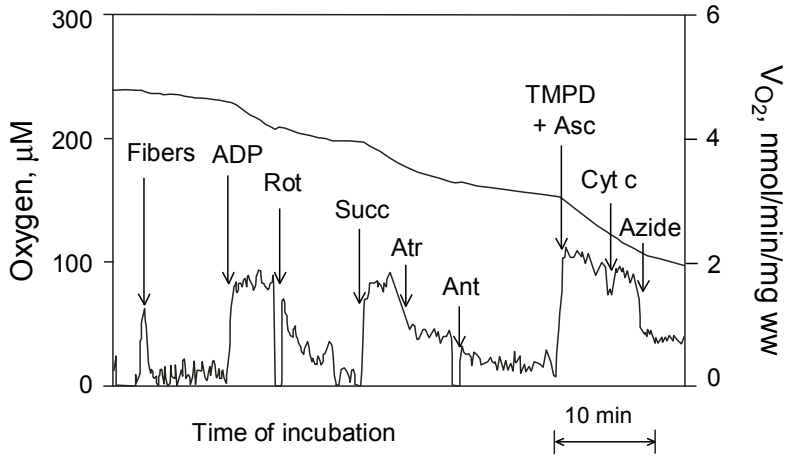

Fig. 1. Investigation of the respiratory chain and oxidative phosphorylation in atrial fibers from the patient with AF. Additions: Fibers, 2 mM ADP, 10 µM rotenone, 10 mM succinate, 0.1 mM ATR, 10 µM antimycin A, 0.5 mM TMPD and 2 mM ascorbate, 8 µM cytochrome c, and 1 mM Na-azide. Here and below the upper line — $[O_2]$, the lower line — V_{O2}.

related glutamate respiration (SRGR) of 119+3% that corresponds to the data in skinned skeletal and cardiac muscles, suggesting normal activity for the complex I (Gellerich et al., 2004, Gellerich et al., 2002). Compared to atria in SR-group of patients the atrial fibrillation was associated with decreased SRGR, this indicating the impairment of the complex I (Gellerich et al., 2004, Gellerich et al., 2002) and increased V_{Succ} and proton leak. Fibrillating atria are characterized by hypertrophy of cardiomyocytes, increased number of abnormally small and elongated mitochondria accumulating between the myofibrils (Ausma et al., 1997, 2000, 2001), and increased tissue content of mtDNA (Lin et al., 2003), these changes suggesting that accelerated mitochondrial biogenesis may contribute to increased succinate-dependent respiration. Based on the current knowledge, several signalling pathways responsible for mitochondrial proliferation can be outlined. First, chronic energy deprivation is associated with activation of AMP kinase which promotes mitochondrial biogenesis and expression of respiratory proteins through activation of nuclear respiratory factor-1 (Bergeron et al., 2001, Zong et al., 2002). AF is also associated with depressed energetical status as the atrial PCr and ATP contents are markedly decreased (Tsuboi et al., 2001, Ausma et al., 2000) and atria with more expressed decrease in tissue [ATP] are more prone to sustained AF (Cha et al., 2003). Because the amplitude of decreases in tissue PCr and ATP levels is sufficient to activate the AMP kinase (Tian, et al., 2001), the pathway mediated by that enzyme may simulate the mitochondrial biogenesis. Secondly, atrial stretch due to volume overload is causal for activation of the mitogen activated protein kinases (MAPK) (Sadoshima et al., 1992, Liang et al,. 1997). The third mechanism may be related to the effects of cardiac glycosides administered to patients with AF to control their ventricular rate. Ouabain, independently of its positive inotropic action activates a multiple growth-related genes either via MAPK or through promoting mitochondrial ROS production, both processes being mediated by GTP-binding protein Ras activation (Liu et al., 2000, Xie et al., 1999). Notably, increased V_{Succ} was not accompanied by corresponding change in V_{Glut} in fibrillating atria which suggests limitation of electron flow at the level of complex I that could be overcome by feeding the respiratory chain via complex II. It is not excluded that it is ROS production via ouabain-stimulated pathway that deteriorates the proteins of the complex I of the respiratory chain directly or through deletions of mtDNA, increased frequency of which in fibrillating atria has been recently documented (Tsuboi et al., 2001, Ausma et al., 2000, Lin et al., 2003). In association to that the increased proton leak observed suggests ROS injury. Disproportional changes in glutamate- and succinate-dependent respiration in fibrillating atria may be part of the myocardial adaptation to increased workload, as suggested by findings that succinate dehydrogenase activity is much more upregulated than glutamate dehydrogenase and malate dehydrogenase under endurance training in skeletal muscles (Oscai et al., 1971). Which of these mechanisms actually plays a role should be clarified in further studies.

Table 1. Characterization of oxidative phosphorylation and its coupling to CK and AK in saponin-skinned atrial fibers. Rates of respiration (V) registered as shown in Fig. 1 are given in nmol O_2/min/mg ww.V_0 — basal respiration without ADP or ATP; V_{Glut} — ADP-stimulated respiration in the presence of glutamate and malate; V_{Succ} — ADP-stimulated respiration in the presence of rotenone and succinate; V_{COX} — activity of cytochrome oxidase, calculated for each individual fiber as [$V_{COX} = (V_{O2TMPD} - V_{O2TMPD+NaN3}) + 0.21$], where V_{O2TMPD} is the respiration rate in the presence of TMPD and ascorbate, $V_{O2TMPD+NaN3}$ is the TMPD-stimulated respiration rates after addition of NaN_3, and 0.21 is an activity of COX in the presence of NaN_3; V_{ATP} — respiration in the presence of glutamate and malate and 50 μM ATP; V_{Atr} — respiration after inhibition of succinate-stimulated respiration by atractyloside; V_{FCCP} — respiration in the presence of uncoupler, FCCP; V_{Cyt-c} — respiration in the presence of FCCP and excess cytochrome c; RCI(Glut) - V_{Glut}/V_0; SRGR - V_{Glut}/V_{Succ}; RCI (Succ) - V_{Succ}/V_{Atr}, and Proton leak — difference between the rates of respiration before and after addition of antimycin A (Fig. 1). I_{AK} and I_{CK} were calculated as the relative stimulation of ATP — dependent respiration with AMP and creatine respectively (Fig. 5). *p<0.05, **p<0.01compared to SR-group. ¤¤¤ p<0.001 — compared to V_{cox} of SR-group. n- number of patients/atria assessed. Here and elsewhere for each of the muscle specimen/patient at least two parallel measurements were performed.

	V_0	V_{Glut}	RCI (Glut)	V_{Succ}	SRGR (%)	RCI (Succ)	Pro-ton leak	V_{COX}	V_{ATP}	I_{AK}	I_{CK}	V_{Atr}	V_{FCCP}	V_{Cyt-c}
SR-group (n=34)														
Mean	0.16	0.94	6.41	0.86¤¤¤	119	1.98	0.3	1.09	0.25	2.18	1.18	0.26	0.91	0.95
SEM	0.01	0.05	0.38	0.03	3	0.05	0.01	0.04	0.01	0.11	0.12	0.02	0.05	0.05
F-goup (n=15)														
Mean	0.19	1.05	5.46	1.04*	104*	2.0	0.37**	1.13	0.28	1.94	0.93	0.25	0.96	0.92
SEM	0.01	0.1	0.35	0.08	3	0.12	0.03	0.09	0.01	0.16	0.10	0.03	0.11	0.07

In a separate set of experiments the parameters of oxidative phosphorylation were compared between human atria and ventricles. Table 2 shows that the rates of ADP dependent respiration in the presence of NADH- (V_{Glut}) or $FADH_2$-linked substrates (V_{Succ}) and the maximal capacity of electron flow (uncoupled respiration in the presence of FCCP) were 3.6 and 3.8 times lower, respectively, in atria than in ventricular muscles. This complies with less intensive oxidative metabolism in atrial myocardium compared to ventricular one (Bass et al., 1993, Anflous et al., 1997, Ventura-Clapier et al., 1998, Vannier et al., 1995). Modest aerobic metabolism appears to be balanced with smaller energy cost, since human atria develop less isometric tension than ventricles (van der Velden et al., 1999). The high value of RCI in the presence of NADH-linked respiratory substrates (6.4 and 5.6 in atria and ventricles, respectively) is comparable with the corresponding parameter in the myocardium of normal rat (Saks et al., 1998, Ventura-Clapier et al., 1998, Vannier et al., 1995). Thus, the mean values of parameters of oxidative phosphorylation appeared to be normal in both types of myocardium studied.

Table 2. Functional properties of mitochondria in skinned fibers of human atria and ventricles. The values for V_0 and V_{ADP} in the presence of glutamate/malate and pyruvate/malate substrate pairs were not different in atrial fibers. Therefore, the corresponding data were pooled into a group assigned as V_{Glut}. The values are given as mean of n (in parentheses) biopsies ± S.D, with 2–4 parallel measurements for each biopsy specimen. * — $p < 0.05$ compared to atria.

	Atria	**Ventricles**
	nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ww	
V_0	0.16 ± 0.01 (34)	0.28 ± 0.08* (9)
V_{Glut}	0.94 ± 0.05 (34)	3.16 ± 1.61* (16)
V_{SUCC}	0.86 ± 0.03 (34)	2.58 ± 1.2* (16)
V_{ATR}	0.26± 0.02 (34)	1.09 ± 0.32* (16)
V_{FCCP}	0.91 ± 0.05 (34)	4.16 ± 1.21* (16)
RCI	6.41 ± 0.38 (34)	5.6 ± 0.5 (9)
$RCI_{SUCC/ATR}$	1.98 ± 0.05 (34)	2.45 ± 0.79 (16)

1.2. Characterization of the energy transfer between mitochondria and ATPases

The activities of CK and AK in atrial homogenates of SR-group (n=25) were 315.4±17.9 and 93.2±5.3 μmol/min g ww, respectively. Of total CK activity the Mi-CK, MM-CK, MB-CK and BB-CK comprised 14.1±0.6, 44.1±0.8, 58.7±0.7 and 2.9±0.2%, respectively. No statistical differences were revealed in kinase activities between the SR and F groups.

To assess the role of mi-CK in atrial mitochondria, the kinetics of stimulation of respiration by exogenously added ADP in the absence and presence of 20 mM creatine was analysed (Fig. 2).

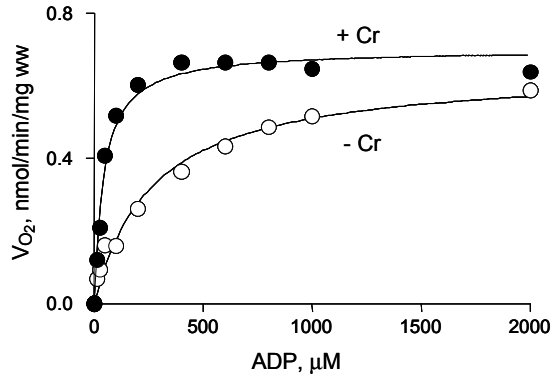

Fig. 2. Effect of creatine (20 mM) on V_{O2} vs [ADP] relationships in skinned atrial fibers of SR-patient with CAD and combined valvular disease. With and without creatine the hyperbolic relationship fitting with the Michaelis-Menten equation was obtained.

In the absence of creatine the apparent Km for ADP and Vmax in SR-group (n=15) were 264.7±31.7 μM and 0.71±0.1 μmol/min/mg ww, respectively. Creatine increased the apparent affinity of mitochondria to ADP, as the Km values decreased to 52.8±4.8 μM (Fig. 2) resulting in creatine index of 5.4±0.9 without change in Vmax (0.73±0.01), that complies with the creatine effects in ventricular myocardium in rat and mice (Seppet et al., 1991, Saks et al., 1998, Braun et al., 2001, Kuznetsov et al., 1996). No differences in kinetics of regulation of respiration either with ADP or creatine were registered between the SR- and F-groups (Results not shown). These results show that in human atrial myocardium, the mi-CK is tightly coupled to ANT, in contrast to function of mi-CK in rat atria, where no coupling has been observed (Anflous et al., 1997, Vannier et al., 1995). The simplest explanation of the low affinity of mitochondria to exogenous ADP in the absence of creatine would be that due to large diameter of the multicellular muscle preparations ADP diffuses within the bulk water from medium into the cells slower than it is phosphorylated by mitochondria, this building up large concentration gradients. Therefore, the skinned diffusion distances for adenine nucleotides were directly measured. Both electron and laser confocal microscopy (Fig. 3) show that after detachment of the fibers by needles and saponin-treatment that removes the sarcolemma but leaves other cellular structures intact, the cardiomyocytes become transversally separated from each other but remain connected longitudinally by intact intercalated disks, thereby forming the multicellular fibers. From these experiments the real diffusion distances for adenine nucleotides were found not to exceed the radius of cardiomyocyte (2.5–10 μm). It has been shown that within these distances adenine nucleotides reach mitochondria faster than they are phosphorylated by oxidative phosphorylation (Saks et al., 2001, Seppet et al., 2004). Thus, the low apparent affinity of mitochondria to exogenous ADP registered in atrial fibers cannot be attributed to the geometry of the skinned fibers, but

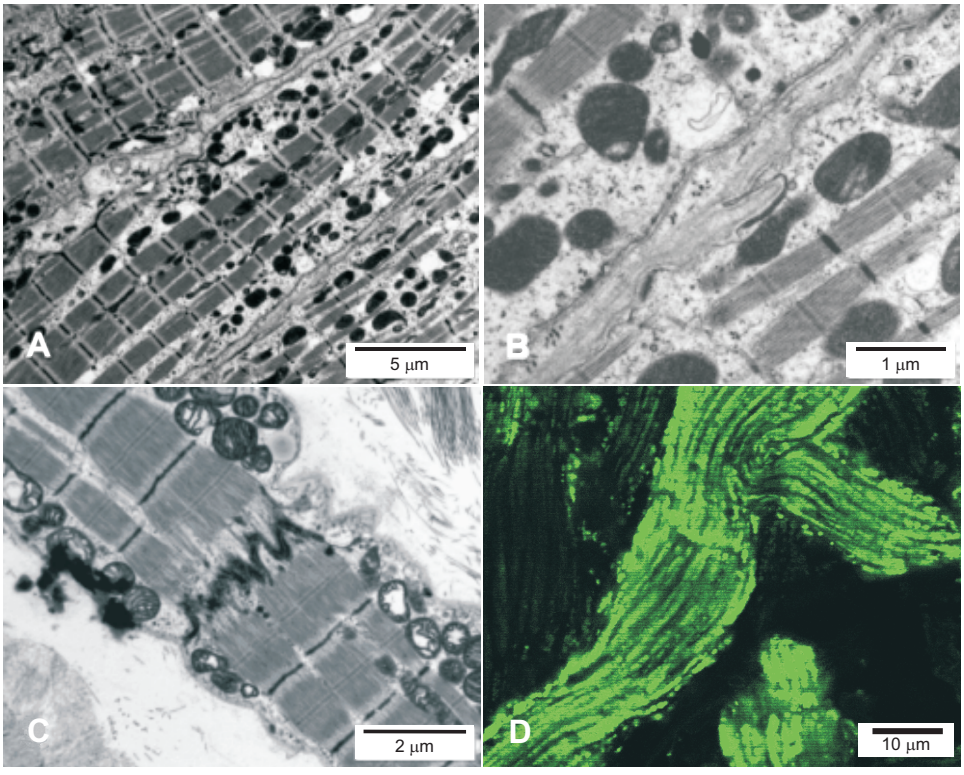

Fig. 3. A, B, C, Electron microscopy of the native (nonpermeabilized) atrial fibers of SR-patient with CAD at lower (A, 4200x) and higher (B, 14000x) magnifications, and of the saponin-permeabilized fibers (C, 14000x). In contrast to native atrial preparations, the saponin-permeabilized fibers are separated from each other and the space between the fibers appears pale suggesting that its constituents are largely washed out during the skinning procedure. The sarcolemma has disappeared, but subsarcolemmal mitochondria and other structures including the intercalated disks are intact. D, Laser confocal imaging of mitochondria in atrial skinned fibers of the patient with AF. Note dissociation of cardiac fibers from each other, but intact intercalated disks and uniform distribution of the Mitotracker Green dye across the cells. The bars scale the distances.

reflects compartmentation of the energy metabolism in the individual cardiomyocytes within the fiber (Saks, et al., 2001, Seppet et al., 2004). Owing to specific organization of energy metabolism, the decreased Km for ADP in the presence of creatine (Fig. 2) suggests effective coupling of mi-CK to ANT, due to which the local [ADP] near ANT increases that results in maximal stimulation of respiration at much lower exogenous [ADP] than without creatine, this explaining the decreased Km for ADP in regulation of respiration (Saks et al., 1998, Saks et al., 2001).

Compared to mi-CK, the kinetics of interaction of mi-AK with oxidative phosphorylation was very different, as activation of respiration by AMP required much less ATP for the maximal effect, than that by creatine. AMP stimulated respiration even without added ATP (Fig. 4) that means that the trace amounts of ATP bound to intracellular structures were sufficient for triggering the respiration-linked ADP-ATP cycling in the mitochondria. The time-dependent increase in respiration at submaximal [AMP] (Fig. 4B, inset) suggests that the stationary [ADP] could not be adjusted due to increasing production of that nucleotide. For that reason it was impossible to estimate the coupling between mi-AK and ANT from the effects of AMP on Km^{ADP} in regulation of respiration. Therefore, the protocol for assessing the stimulatory effects of AMP at submaximal [ATP] on respiration expressed as the AK index (I_{AK}) was developed and applied along with registration of analogous index (I_{CK}) that characterizes coupling of mi-CK to ANT in the same muscle fiber (Fig. 5). After registration of the basal respiration rate (V_0), 50 μM ATP was added to achieve a very limited rate of ADP-ATP cycling between the mitochondria and ATPases. The addition of 2 mM AMP markedly augmented the respiration over its basal value due to ADP production by the mi-AK, and this process was abolished by AP_5A, a AK inhibitor. Effective termination of mi-AK reaction allowed to evaluate the stimulation of respiration by creatine, due to interaction between the mi-CK and oxidative phosphorylation. Table 1 shows a twice larger stimulation of respiration at 50 μM ATP with AMP than with creatine in all muscle groups.

Stimulation of respiration by creatine or AMP (Fig. 5) may result from increased cytoplasmic [ADP] produced by activation of the MM-CKs or AK_1 outside of mitochondria — in myofibrils and at the sarcoplasmic reticulum. Alternatively, the respiration could be upregulated by endogenous ADP produced near ANT owing to its functional coupling to mi-CK or mi-AK (Braun et al., 2001, Seppet et al., 2001, Saks et al., 2001, Kaasik et al., 2001).

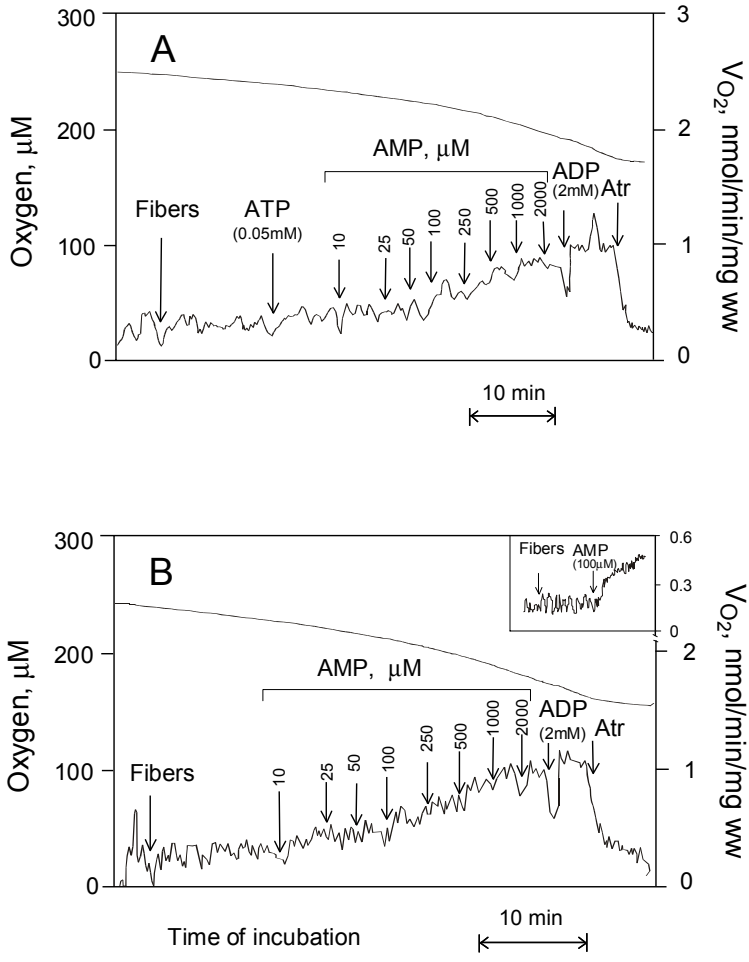

Fig. 4. Respirometric investigation of the coupling of mi-AK to oxidative phosphorylation in atrial fibers from the SR patient with CAD by applying increasing concentrations of AMP in the presence (A) or absence (B) of 50 µM ATP.

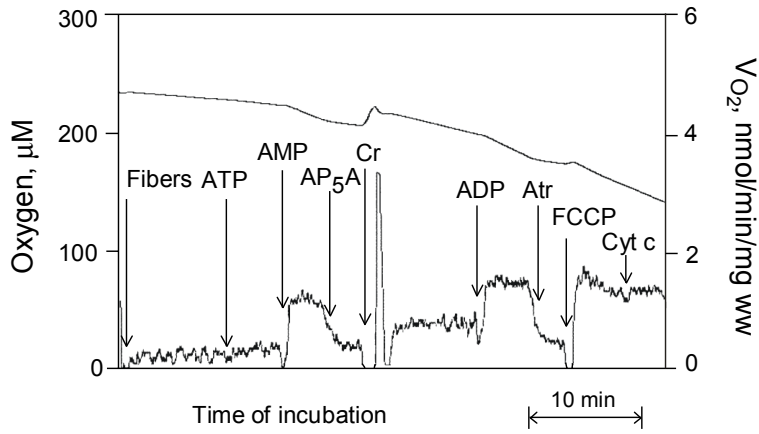

Fig. 5. Respirometric investigation of coupling of kinases to oxidative phosphorylation in atrial fibers from the patient undergoing the GABG. The V_{O2} was measured in the presence of 10 mM glutamate and 2 mM malate. Additions: Fibers, 50 µM ATP, 2 mM AMP, 0.2 mM AP_5A, 20 mM Cr, 2mM ADP, 0.1 mM ATR, 1 mM FCCP, and 8 µM cytochrome c.

To discriminate between these alternatives in human atria, the effects of creatine and AMP on respiration were assessed in the presence of saturating concentrations of PK (20 IU/ml) and PEP (5 mM) that effectively eliminates the cytosolic ADP (Seppet et al., 2001, Seppet et al., 2001, Kay et al., 2000). PK in the presence of PEP negligibly inhibited the respiration stimulated by 50 µM ATP, whereas creatine stimulated it above the levels registered before PK addition (Fig. 6). Addition of ATP (2 mM) augmented the creatine-stimulated respiration, suggesting that 50 µM of [ATP] was not fully activating the mi-CK due to its high Km^{ADP} value (Fig. 2). Because of the differences compared to creatine in the kinetics with respect to ATP 2 mM AMP yielded the maximally possible levels of respiration already at 50 µM ATP (Fig. 6). As with creatine, the PK+PEP system was unable to abolish the AMP-caused stimulation of respiration. Thus, either with creatine or AMP, it was not cytoplasmic ADP diffusing to ANT but endogenous ADP generated near the ANT that strongly stimulated the oxidative phosphorylation.

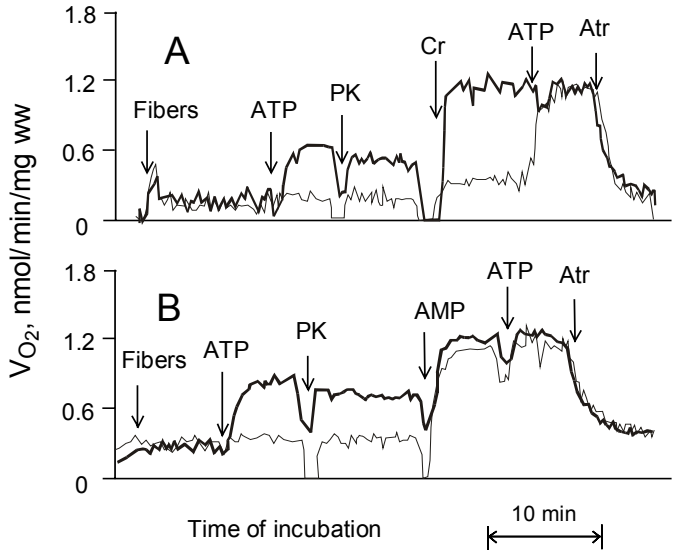

Fig. 6. Respirometric investigations of coupling of kinases to oxidative phosphorylation in atrial fibers from the patient surgically operated for aortic valve replacement. Additions: A, (thin line) — fibers, 50 μM ATP, 20 IU/ml PK, 20 mM Cr, 2 mM ATP, 0.1 mM atractyloside. Thick line — the same, but 2 mM ATP instead of 50 μM ATP. B, (thin line) — fibers, 50 μM ATP, 20 IU/ml PK, 2 mM AMP, 2 mM ATP, 0.1 mM atractyloside. Thick line — the same, but 2 mM ATP instead of 50 μM ATP. Note the effective control over the oxidative phosphorylation at the level of ANT (inner mitochondrial membrane) revealed by atractyloside.

Principally it is possible that mitochondria and ATPases exchange ATP and ADP directly, without CK- or AK-phosphotransfer systems. Fig. 7 demonstrates the results of the experiments performed to test the existence of direct ADP transfer between the ATPases and mitochondria in human atria. At first, all ATPases (CaMgATPases, for the medium contained both Ca^{2+}- and Mg^{2+}-ions) in SR and myofibrils were activated by addition of 2 mM of ATP. The ADP flux produced by ATPases entirely passed through the PK reaction, which also clamped the [ATP] in the medium (Fig. 7A,B,C). Launching of oxidative phosphorylation by respiratory substrates resulted in rapid decrease in the flux, whereas atractyloside exerted the opposite effect, these changes indicating the utilization of ADP during oxidative phosphorylation. The mitochondrially phosphorylated but inaccessible to PK+PEP system ADP flux (direct ADP transfer) was found to be 1.18 $\pm$ 0.09 nmol/min/mg ww, i.e. comprising 55 $\pm$ 2% of the maximal ADP flux. The same experiment was continued to estimate the function of MM-CK in the ATPase's end of the CK-phosphotransfer

network, by adding 20 mM PCr into the medium after atractyloside. It can be seen that under PCr the ADP flux available to PEP+PK system immediately decreased (by 66 %) compared to that without PCr (Fig. 7 B,C). This indicates functional coupling between MM-CK and ATPase reactions due to which ADP produced by ATPases is rephosphorylated by PCr, and therefore unaccessible to cytoplasmic PK.

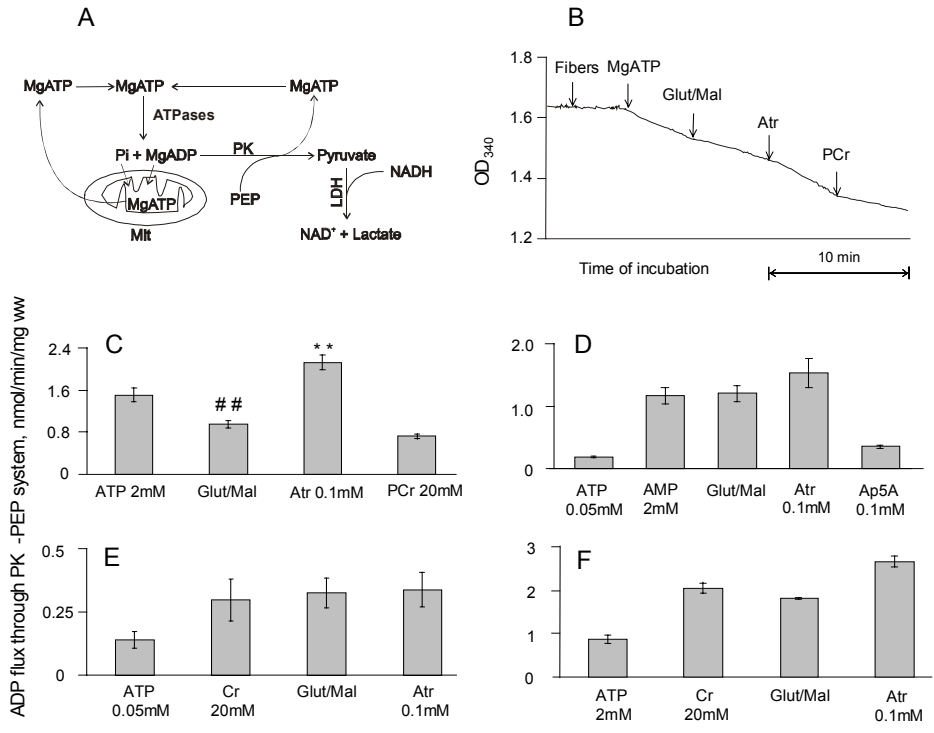

Fig. 7. A, The principle of competition between mitochondria and cytoplasmic PK+PEP system for phosphorylation of ADP produced by the ATPases. B, Original trace of registration of the ADP flux through the PK reaction in the presence of 5 mM PEP and coupled LDH system in atrial fibers from the patients undergoing the CABG. Ordinate — optical density at 340 nm equivalent to [NADH] in the medium. C, Quantitation of the direct transfer of ADP from ATPases to mitochondria and coupling of MM-CK to ATPases. In this experiment the atrial fibers from SR patients (n=11) were used. ## — $p<0.01$ compared to value without substrates;**$p<0.01$ compared to parameter before atractyloside addition and after PCr addition. D, Estimation of the AK-phosphotransfer system at low [ATP] (50 μM). In this experiment the atrial fibers from 6 SR patients with valvular disease were used. E, Estimation of the CK-phosphotransfer system at low [ATP] (50 μM). F, Estimation of the combined function of CK-phosphotransfer system and direct adenine nucleotide transfer at high [ATP] (2 mM). In E-F, the atrial fibers from 3 SR patients with valvular disease were used.

At low [ATP] (50 μM) only a limited ADP flux from ATPases was produced (Fig. 7D) this being responsible for minimal respiratory activation (Fig. 6). However the addition of 2 mM AMP gave as much ADP as in the presence of 2 mM ATP alone (Fig. 7C). Creatine in the presence of 50 μM ATP (Fig. 7E) produced 4-times less ADP than AMP that explains the smaller effect of creatine on respiration compared to AMP (Table 1) as well. In both cases, with AMP and creatine, neither respiratory substrates nor atractyloside exerted effect (in absolute or relative terms) on the ADP flux. Hence, contrary to that in the presence of 2 mM ATP alone, the direct transfer did not manifest at low [ATP]. When both the ATPases and CK were activated by 2 mM ATP and 20 mM creatine (Fig. 7F), the total ADP flux largely exceeded that in previous experiments (Fig. 7,C,D,E), and the mitochondria became to control the ADP available for PK. Notably, now the direct flux was less (32 $\pm$ 2%) than in conditions when exclusively the ATPases provided ADP for mitochondria (55%, $p<0.001$, Fig. 7B,C), because the MM-CK consumed part of ADP produced by ATPases.

2. Mathematical modeling (Paper III)

The results described above show that the stimulation of mitochondrial respiration strongly depends on the source of ADP: ADP generated endogenously by ATPases, mi-CK or mi-AK is much more effective than ADP added exogenously. On the other hand, the PK+PEP system is unable to compete with mitochondria or MM-CK for ADP produced in ATPase reactions. These data support the hypothesis that in oxidative muscle cells the ATPases and mitochondria are organized into functional complexes, the ICEUs that are isolated from cytoplasm (Seppet et al., 2001, Saks et al., 2001). How such complexes are formed, is still not clear. There are two promising ways to address this issue; one based on analyse the potential effect of ICEUs on diffusion of adenine nucleotides in the muscle cells by comparing the results of experimental kinetic measurements with the solutions of different mathematical models. Another way is to study the role of different cytoskeletal proteins in determining the nature of interaction between mitochondria and ATPases.

While mathematical modeling approach was applied, two different mechanisms of diffusion restriction for adenosine was assumed. One mechanism is based on the molecular crowding that results in uniform diffusion restriction (DistICEU model, Fig. 8) that is proportional to the diffusion distance, i.e. half of the cardiac cell diameter. Another restriction must arise from local diffusion barriers created by the borders of the ICEUs (localized or nonuniform diffusion restriction, SmallICEU) (Fig. 8).

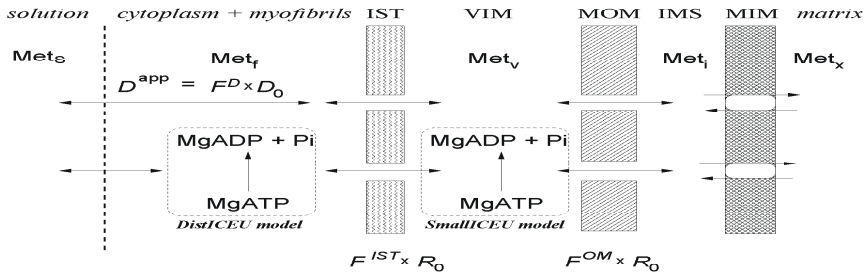

Fig. 8. Schematic presentation of ATP, ADP and Pi diffusion pathways from solution into mitochondria. Met_s, Met_f, Met_v, Met_i and Met_x — metabolite concentrations in the solution, in the myofibrillar and cytoplasmic compartment, in the vicinity of the mitochondria (VIM), in the mitochondrial intermembrane space (IMS), and in mitochondrial matrix, respectively. IST, MOM and MIM — intracellular structures between cytoplasm and VIM, mitochondrial outer and inner membrane, respectively. Depending on the model used, ATP is hydrolyzed either in VIM or myofibrils. Diffusion pathway is characterized by three model parameters: F^D, F^{IST}, and F^{OM}.

To distinguish between these two types of restrictions the experimental data were compared with the data obtained by the mathematical model for the following processes (i) mitochondrial respiration rate dependency on exogenous ADP and ATP concentrations, (ii) the inhibition of endogenous ADP-stimulated respiration by PK+PEP system competing with mitochondria for ADP, (iii) the kinetics of oxygen consumption stabilization after the addition of 2 mM ATP or ADP, (iv) the ADP concentration buildup in the medium after the addition of ATP, and (v) the ATPase activity with inhibited mitochondrial respiration. The results show that when the intracellular diffusion was assumed to be distributed uniformly (DistICEU model, Fig. 8), only the measuremens of the respiration rate as a function of exogenously added ADP or ATP (Fig. 9) and PK+PEP system inhibition of the respiration (not shown) were reproduced by the mathematical model.

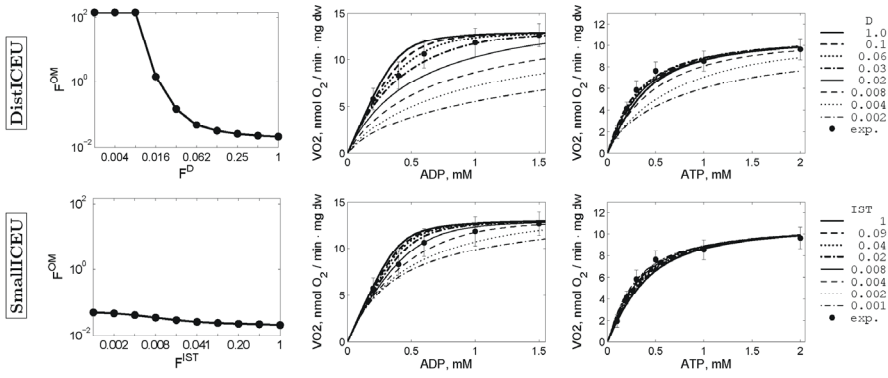

Fig. 9. Mitochondrial outer membrane permeability (indicated by F^{OM}) as a function of the diffusion restriction at the ICEU level (F^D or F^{IST} depending on the model) found by fitting the measured VO_2-ADP and VO_2-ATP relationships. The ICEU was simulated by two models: DistICEU solution is shown in the upper row, SmallICEU — in the lower row. The results of the fitting are presented by F^{OM}-ICEU level diffusion restriction relationship (the left column), and VO_2-ADP, ATP relationships (the middle and the right columns). The computed VO_2 are shown by lines (see the legend on the right for corresponding ICEU level restriction, "D" and "IST" stand for F^D and F^{IST}, respectively), experimental measurements (exp) are adapted from Saks et al., 2003. Note that the increase of the diffusion restriction at the ICEU level increases optimal F^{OM} value for both models and the model solutions are not able to reach measured VO_2 if the used ICEU restriction is too large (F^D or F^{IST} is very small, the middle column).

In contrast, when the localized diffusion restriction in the space containing ATPases in the near proximity of the mitochondria was assumed (SmallICEU model, Fig. 8), all the five measurements were reproduced by the model (Figs 9 and 10 depict part of these results). These data show that diffusion restrictions of metabolies are not distributed uniformly within cardiac muscles but are localized in certain areas. The non-uniform distribution of diffusion restriction in cardiac muscle cells is in concord with the results of many studies demonstrating that the apparent Km for exogenous ADP of the mitochondrial respiration depends on the structural organization and intactness of membranous structures, i.e. localized diffusion restrictions. The hypoosmotic treatment of skinned cardiac fibers, which results in the appearance of the population of mitochondria with the disrupted outer membrane, changes the kinetics of mitochondrial respiration to biphasic, attributable to two populations of mitochondria: apparent Km for exogenous ADP ~300 μM and ~30 μM corresponding to mitochondria with intact and disrupted outer membrane, respectively (Saks et al., 1993, Saks et al., 1995b). Secondly, the similar

biphasic kinetics of the respiration has been recorded in skinned cardiac fibers of transgenic desmin-deficient mice (Kay et al., 1997), when also two populations of muscle cells with normal and disintegrated structure became apparent. Such a sensitivity of the apparent Km for ADP of the mitochondrial respiration to the hypoosmotic treatment and structural organization can be attributed to changes in permeability of the porin pores in the mitochondrial outer membrane (Saks et al., 1994, Kay et al., 1997), or as suggested by our analysis, it could be the permeability of ICEU structures that is affected by the hypoosmotic treatment and by changes in structural organization of the cell.

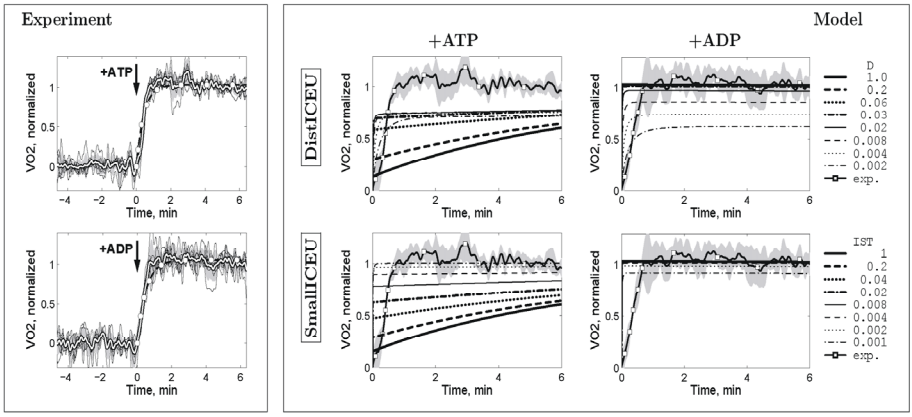

Fig. 10. Analysis of VO_2 stabilization after addition of 2mM ATP or ADP. Experiment: Addition of ATP (ADP) is indicated by an arrow, time shown on x-axis is relative to the time-moment of ATP (ADP) addition. Individual oxygraph tracings are shown by thin solid lines (n=6); mean value at every time-moment is presented by thick white solid line; gray area corresponds to mean value ± standard deviation obtained for each time-moment. The following time-constants were obtained from fitting mean VO_2 by single exponent: 25s and 30s for VO_2 traces after ATP and ADP addition, respectively. The exponents are shown by dashed thick lines with open dots. Model: VO_2 stabilization was computed by the both models (DistICEU in the upper row, SmallICEU in the lower row) and compared with the measurements. The computed stabilization of VO_2 is rapid after 2mM ADP addition in correspondence with the measurements. However, after the addition of 2mM ATP, VO_2 stabilization is strongly dependent on the used ICEU level restriction (see the legend for F^D and F^{IST} values marked by "D" and "IST", respectively). The measured VO_2 is presented by mean and gray area indicating mean value ± standard deviation region.

3. Energy metabolism in oxidative muscles of dystrophin deficient mice (Paper I)

To modify the function of ICEUs by altering cytoskeletal organization of the cellular structure, the dystrophin-deficient mice were generated and the mitochondrial function in skinned cardiac and *m. soleus* fibers were analysed in relation to morphological assays. We found that the lack of dystrophin had no effect on ultrastructure as visible by confocal microscopy, maximal capacity of oxidative phosphorylation, nor on coupling between oxidation and phosphorylation. Addition of 20 mM creatine to the medium brough about a decrease in K_m for ADP in wild-type ventricles and *m. soleus* (Fig. 11), which suggests normal functional coupling between mi-CK and ANT as in Fig. 3. However, in the same muscles of MDX mice creatine suppressed the K_m for ADP to a lesser extent that resulted in a lower creatine index than in the corresponding muscles of the wild-type mice. Thus, the lack of dystrophin attenuated the control of respiration by creatine in slow-twitch oxidative muscles. Since the total creatine kinase activity of dystrophin-deficient ventricles was similar to that in control hearts (206.8±22.6 and 235.3±14.3 μmoles/min g ww, respectively, with markedly increased proportion of mi-CK (44.4±3.6 and 33.0±2.7%, respectively, $p<0.05$), the decreased mitochondrial response to creatine (Fig.11) could not be attributed to changes in mi-CK activity. It is known that the muscles of MDX mice exhibit abnormally high rates of ROS production (Hauser et al., 1995) that entails peroxidation of cardiolipin (Paradies et al., 1998), a component of the MIM binding the mi-CK (Schlattner et al., 1998). Therefore, impaired binding of mi-CK to cardiolipin due to its peroxidative modulation may attenuate the coupling of mi-CK to ANT.

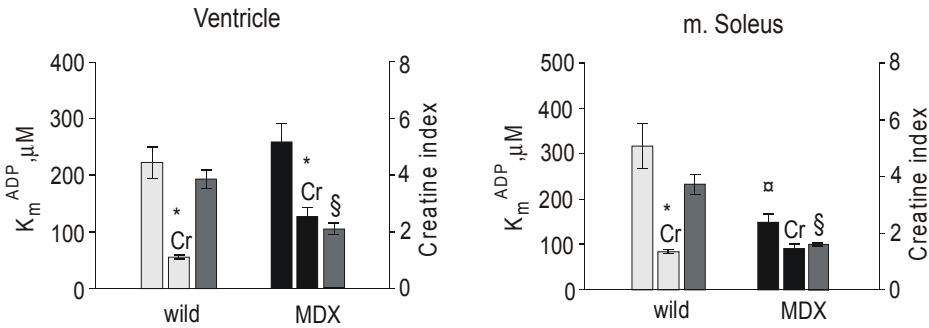

Fig. 11. Effect of 20 mM creatine on mitohondrial affinity to ADP (K_m^{ADP}). K_m^{ADP} without creatine/ K_m^{ADP} with creatine = creatine index (grey columns). Cr- in the presence of creatine. * $p<0.05$ compared to K_m^{ADP} without creatine; ¤ $p<0.05$ compared to K_m^{ADP}corresponding parameter in wild type muscle group. § $p<0.05$ compared to creatine index in wild-type muscle group.

Fig. 11 also demonstrates that compared to normal *m. soleus* the K_m for ADP in regulation of respiration in the absence of creatine was decreased in MDX counterpart. A similar phenomenon, attributed to decreased barrier function of MOM which allows the mitochondrial respiration to be controlled by cytosolic fluctuations of [ADP], has been observed in the oxidative muscles after genetic or chemical modification of the creatine kinase system (Veksler et al., 1995, Clark et al., 1994) and in cardiomyocytes of the desmin-deficient mice (Kay et al., 1997). These facts strongly suggest that the decreased K_m for ADP (Fig. 12), together with increased cellular [ADP] (Kemp et al., 1993, Lodi et al., 1999) can be taken to indicate the increased role of the ADP diffusion in linking ATPases with mitochondria in *m. soleus* in response to the lack of dystrophin. To ensure the effective ADP diffusion to ANT, [ADP] must be higher in the cytosol and lower in the intermembranous space. However, high cytosolic [ADP] decreases the free energy of ATP hydrolysis (Ventura-Clapier et al., 1994), whereas low [ADP] near ANT limits the rate of oxidative phosphorylation. Within these constraints, increased permeability of the MOM is beneficial as it confers decreased ADP concentration gradient (Gellerich et al., 1993). Nevertheless, a shift of high [ADP] to the intermembrane compartment may then result in the following: firstly, direct activation of respiration may occur that may explain creatine's inability to alter the K_m for ADP. Secondly, it may destabilize the mi-CK forward reaction, which results in abnormally low [PCr] and reduced muscle work, especially at higher workloads (Aliev and Saks, 1997). It follows thus that excessive entry/deposition of ADP into the intermembranal space may represent either an independent factor capable to compromise the PCr synthesis or it may complicate the influences of other factors such as imbalanced expression of mi-CK and ANT and/or oxyradical defects in mi-CK described above.

Considering the existence of direct exchange between mitochondria and ATPases (Fig. 7), it was interesting to assess whether this system may be affected by the lack of dystrophin. To answer that question, we analysed the competition between mitochondria and the powerful PEP-PK system (with an activity exceeding that of total ATPase more than hundred times) for ADP produced in ATPase reactions similarly to studies depicted in Fig. 7. The results show that the mitochondria effectively competed with exogenous PK+PEP system for ADP produced by ATPases in murine ventricles. Analysis of the full set of analogous experiments showed that after switching on the processes of oxidative phosphorylation the flux through the PK-PEP system became inhibited by 35% in wild-type cardiac fibers (Fig. 12A). Thus, upon induction of oxidative phosphorylation this fraction of total ADP flux was compartmentalized within the space which included both mitochondria and ATPases together with some fraction of adenine nucleotides, but could not be controlled by the PK-PEP system. From these experiments the absolute ADP flux taken up by mitochondria was calculated to be about 1 μmoles/min/g wwt in wild-type muscles (Fig. 12B). When the same parameter was estimated for MDX mice

ventricles, a marked increase in the mitochondrial ADP phosphorylation was revealed compared to the wild-type muscle group (Fig. 12B).

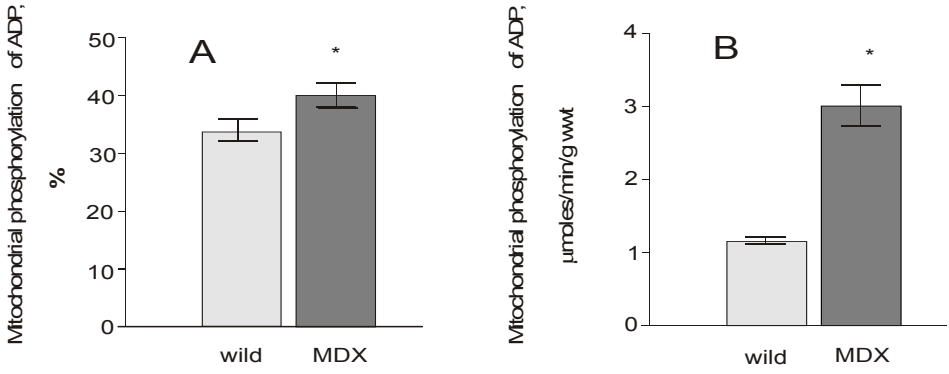

Fig. 12. Comparison of the mitochondrial phosphorylation of ADP in relative (A) and absolute (B) terms in cardiac fibers of the MDX and wild-type mice. The percentage of mitochondrial phosphorylation of ADP corresponds to this fraction of the total ADP flux that is inhibited after switching on the respiration by adding the substrates. The absolute flux is a difference between the rates of the ADP phosphorylation by PK-PEP system in the absence and presence of glutamate and malate. * $p<0.05$ compared to corresponding parameter in wild-type myocardium.

The lack of dystrophin attenuates or stimulates the expression of number of the proteins, such as titin, troponin I, α-tubulin and Rac1 which participate in structural organization of the muscle cells (Tkatchenko et al., 2000). Based on observation, that about 40% of dystrophin is tightly bound to the contractile apparatus (Meng et al., 1996), and that a loss of that dystrophin fraction is critical for the development of cardiac insufficiency (Stromer 1998), the increased mitochondrial uptake of ADP can be taken to suggest the altered cytoskeletal control over the function of the complexes between mitochondria and ATPases.

Altogether the results of this study demonstrate that in the cardiac ventricular and atrial myocardium the mitochondria and ATPases behave as if they were included into functional complexes, termed as the ICEUs (Seppet et al., 2001, Saks et al., 2001) (Fig. 13). The ICEUs compartmentalize part of cellular adenine nucleotides for being used in specialized energy transfer networks (Seppet et al., 2001, Saks et al., 2001), such as CK- and AK-phosphotransfer networks and direct channeling of ATP and ADP. To be effective, this phosphotransfer requires a tight functional coupling between mi-CK and ANT, favouring maximal activation of oxidative phosphorylation despite limited

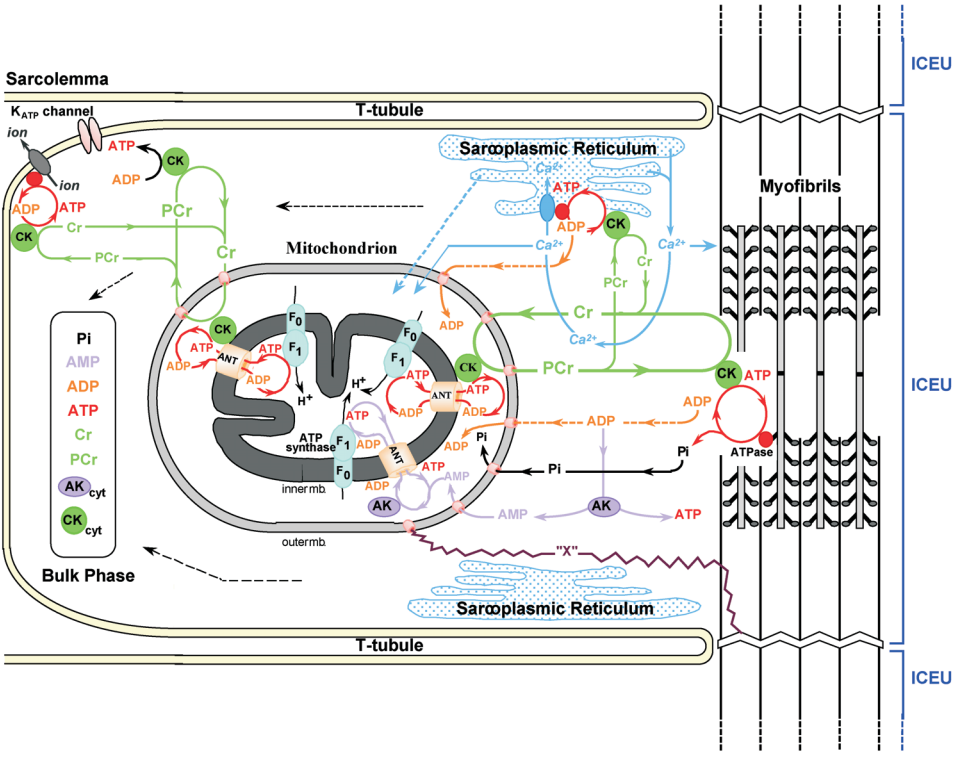

Fig. 13. Schematic presentation of functional ICEUs in the cardiomyocyte. By interaction with cytoskeletal elements, the mitochondria and SR are precisely fixed with respect to the structure of sarcomere of myofbrils between two Z-lines and correspondingly between two T-tubules. Ca^{2+} is released from the SR into the space of the ICEU in the vicinity of the mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within the ICEU do not equilibrate rapidly with adenine nucleotides in the bulk-water phase (cytoplasmic phase). The mitochondria and ATPases of SR and myofibrils are interconnected by metabolic channeling of reaction intermediates and energy transfer via CK- and AK-phosphotransfer networks and direct ATP/ADP exchange. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within the ICEU and bulkwater phase may be connected by some more rapidly diffusing metabolites as Cr and PCr. Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, Pi or PCr) and/or synchronized release of Ca^{2+} during the excitation-contraction coupling process (with permission).

diffusion of cytoplasmatic ADP through the porin channels of MOM (Saks et al., 2001, Saks et al., 2004, Kay et al., 2000) and without the necessity to accumulate the ADP in the cytoplasm. This explains the decreased Km for exogenous ADP in the presence of creatine (Fig. 2) and stimulation of respiration by AMP.

The observation that powerful exogenous PK+PEP system was unable to inhibit stimulation of respiration by creatine and AMP, strongly suggests a compartmentation of endogenous pool of adenine nucleotides within the ICEUs (Fig. 13) where the high-energy-phosphoryl transfer takes place. Probably such a trapping of adenine nucleotides and enzymes for energy transduction occurs due to the cytoskeleton (Andrienko et al., 2003) which links mitochondria to adjacent sarcomere and SR tubules, as suggested by increased affinity of mitochondrial respiration to ADP in skinned oxidative fibers in response to lack of desmin (Kay et al., 1997) or dystrophin (this study). The best explanation of these results would be that cytoskeletal proteins create localized barriers (the protein factors "X" in fig. 13) for movement of adenine nucleotides either within the ICEUs at the level of mitochondrial outer membrane or at the borders of the ICEUs. These barriers form a basis for localized diffusion restriction in cardiac muscle cells which explain the apparent Km for exogenous ADP of the mitochondrial respiration.

AK-phosphotransfer network that may provide a very effective feedback signal between ATPases and mitochondria in conditions of compartmentalized [ATP] decrease and [AMP] increase (i.e. in ischemic or failing heart) (Dzeja and Terzic, 2003, Dzaja et al., 1998, Dzeja et al., 1999, Pucar et al., 2000). High effeciency of AK system may be based on its capability to time-dependently generate ADP from minute amounts of ATP given that AMP is available. This property leaves the AK system operable when the CK system become inhibited.

CONCLUSIONS

1. In human atria the energy metabolism is highly compartmentalized within the functional complexes between mitochondria and ATPases — the ICEUs. The ICEUs capture a part of the cytoplasmic pool of adenine nucleotides for being involved in energy transfer via CK- and AK-networks and/or direct exchange.
2. The functional coupling of mi-AK with ANT is characterized by higher efficiency to stimulate respiration than coupling between mi-CK and ANT in the presence of low levels of cellular ATP.
3. The diffusion restrictions of adenine nucleotides are not distributed uniformly within cardiac muscle cells, but are localized in certain areas. This suggests existence of organized structural barriers controlling the distribution of adenine nucleotides between the cytoplasm to mitochondria. Presumably, the MOM and the borders between serially positioned ICEUs at the levels of each sarcomere represent the structures most strongly limiting the diffusion of adenine nucleotides, whereas the cytoplasm exposes no significant restrictions for that process. Thus, due to existence of ICEUs, the exogenous ADP cannot easily reach the mitochondria to stimulate respiration unless applied in very large concentrations, which results in high value of apparent Km for this nucleotide.
4. Defects in oxidative phosphorylation, appearing as increased proton leak and impaired complex I of the respiratory chain suggest that impaired ATP production probably contributes to sustained AF.
5. The dystrophin-deficient MDX mice exhibit a special myopathy which is characterized by normal oxidative phosphorylation with impaired coupling between the mi-CK and ANT and increased transfer of ADP from ATPases to mitochondria in slow-twitch oxidative muscles. As these properties are associated with defective energy transport they may constitute a basis for development of the muscle weakness in patients with DMD or BMD.

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SUMMARY IN ESTONIAN

Mitokondrite ja ATPaaside interaktsioon oksüdatiivses lihasrakus normis ja patoloogias

Permeabiliseeritud membraaniga (skinneeritud) lihaskiududel teostatud uuringud on näidanud, et kiiretel glükolüütilistel lihastel on võrreldes aeglaste oksüdatiivsete lihastega kõrgem afiinsus eksogeense ADP suhtes hingamise regulatsioonis, mis viitab lihastüübi-spetsiifilisele rakuhingamise regulatsioonile. Nende tulemuste põhjal on hiljuti esitatud hüpotees, et oksüdatiivsetes lihasrakkudes moodustavad mitokondrid ja ATPaasid funktsionaalseid komplekse — rakusiseseid energeetilisi üksuseid (RSEÜ). RSEÜ-d kompartmentaliseerivad osa rakusisestest adeniinnukleotiididest nii, et nad on efektiivselt kasutatavad energiaülekande võrkudes viisil, mis tagavad oksüdatiivse fosforüülimise stimuleerimise ilma adeniinnukleotiidide ja PCr kontsentratsioonide muutusteta tsütoplasmas (metaboolse stabiilsuse). Käesoleval ajal ei ole selge tsütoskeleti valkude roll RSEÜ-de struktuuri moodustamisel, samuti pole tõestatud nende üksuste olemasolu inimese müokardis. Antud töös uuritakse neid probleeme, kasutades permeabiliseeritud lihaspreparaate inimese südame kodadest ja vatsakestest ning düstrofiinipuudusega hiirte lihastest, respiromeetriat, ensüümide kineetilist analüüsi, genoomi modifikatsiooni ning elektron- ja laserkonfokaalmikroskoopiat.

Töö eesmärgid olid:

1. Iseloomustada inimese südame koja CK- ja AK- vahendatud energiaülekande mehhanisme.
2. Võrrelda inimese südame koja ja vatsakese oksüdatiivseid parameetreid.
3. Demonstreerida, et CK- ja AK-fosfaadiülekande võrgud ning adeniinnukleotiidide otsene ülekanne on kompartmentaliseeritud RSEÜ-desse.
4. Iseloomustada energeetilise metabolismi muutusi inimese kojas kodade virvenduse tingimustes.
5. Kasutades MDX-hiirte lihaste skinneeritud kiude, näidata et düstrofiinil on mitokondrite funktsiooni regulatsioonis oluline roll.
6. Võrrelda kahe võimaliku mehhanismi (ühtlaselt jaotunud ja mitokondrite lähedal lokaliseeritud difusioonitakistus) rolli Ca,MgATPaaside ja mitokondrite funktsionaalne seose tekkes skinneeritud südamekiududes.

Töö tulemused võimaldavad teha järgmisi järeldusi:

1. Inimese südame kojas on energeetiline metabolism kompartmentaliseeritud funktsionaalsetesse mitokondrite ja ATPaaside vahelistesse üksustesse – RSEÜ-desse, mis kasutavad osa tsütoplasmas paiknevaid adeniinnukleotiide energia ülekandeks CK- ja AK-võrgustike ja/või nukleotiidide otsese vahetuse kaudu.

2. mi-AK ja ANT vaheline funktsionaalne seos hingamise stimuleerimises on efektiivsem kui mi-CK ja ANT vaheline seos rakusisese ATP madala taseme puhul.
3. Südamerakkudes ei ole adeniinnukleotiidide difusioonitakistus jaotunud ühtlaselt, vaid lokaliseeritult teatud aladesse. Selline olukord viitab organiseeritud struktuursete barjääride olemasolule, mis kontrollivad adeniinnukleotiidide jaotumist mitokondrite ja tsütoplasma vahel. Oletatavasti kujutavad mitokondrite välismembraan ja iga sarkomeeri tasemel kõrvuti paiknevate RSEÜ-de piirid endist neid struktuure, mis kõige tugevamalt takistavad adeniinnukleotiidide diffusiooni, samas kui tsütoplasma ei oma märgatavat mõju diffusiooni takistusele. RSEÜ-dest tingitud takistuse tõttu jõuab eksogeenne ADP mitokondritesse ning stimuleerib hingamist ainult väga suurte kontsentratsioonide juures, mis väljendub kõrges Km väärtuses selle nukleotiidi jaoks.
4. Oksüdatiivse fosforüülimise muutused, mis väljenduvad suurenenud prootonlekkes ja hingamisahela I kompleksi pidurduses, viitavad sellel, et ATP produktsiooni haired võivad kaasa aidata püsiva kodade virvenduse tekkele
5. Düstrofiini puudusega hiirtel kujuneb müopaatia, mida iseloomustavad normaalne oksüdatiivne fosforüülimine koos häiritud mi-CK ja ANT vahelise funktsionaalse seosega ning ADP suurenenud ülekandega ATPaasidest mitokondritesse aeglastes oksüdatiivsetes lihastes. Kuna need omadused on kahjustavad energia transporti, võivad nad olla Duchennei või Beckeri lihasdüstroofiat põdevate patsientidel lihasnõrkuse aluseks.

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Publications

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Peamisteks uurimisvaldkondadeks on südame- ja skeletilihaste rakusisese energiatranspordi mehhanismide uurimine normis ja erinevate patoloogiate tingimustes in vivo.

Publikatsioonid

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