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Cardiac system bioenergetics: metabolic basis of the Frank-Starling law

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The fundamental principle of cardiac behaviour is described by the Frank-Starling law relating force of contraction during systole with end-diastolic volume. While both work and respiration rates increase linearly with imposed load, the basis of mechano-energetic coupling in heart muscle has remained a long-standing enigma. Here, we highlight advances made in understanding of complex cellular and molecular mechanisms that orchestrate coupling of mitochondrial oxidative phosphorylation with ATP utilization for muscle contraction. Cardiac system bioenergetics critically depends on an interrelated metabolic infrastructure regulating mitochondrial respiration and energy fluxes throughout cellular compartments. The data reviewed indicate the significance of two interrelated systems regulating mitochondrial respiration and energy fluxes in cells: (1) the creatine kinase, adenylate kinase and glycolytic pathways that communicate flux changes generated by cellular ATPases within structurally organized enzymatic modules and networks; and (2) a secondary system based on mitochondrial participation in cellular calcium cycle, which adjusts substrate oxidation and energy-transducing processes to meet increasing cellular energy demands. By conveying energetic signals to metabolic sensors, coupled phosphotransfer reactions provide a high-fidelity regulation of the excitation–contraction cycle. Such integration of energetics with calcium signalling systems provides the basis for ‘metabolic pacing’, synchronizing the cellular electrical and mechanical activities with energy supply processes.

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Living cells are open systems exchanging energy and metabolic substrates and products with the environment (Westerhoff & Van Dam, 1987; Nicholls & Ferguson, 2002). This allows cells to perform all the work needed for their biological functions, and to keep entropy low to maintain a high and specific structural organization (Schroedinger, 2000). Evolution has selected ATP along with ADP and P_i as the main system for free energy conversion in cells – a high ratio of $[ATP]/[ADP] \times [P_i]$ makes available free energy both for cellular work and anabolic reactions (Garlid, 2001; Nicholls & Ferguson, 2002). This high ratio is maintained by mitochondrial oxidative phosphorylation and by glycolysis. How the free energy transduction and energy supply/demand balance is maintained and which signals and mechanisms ensure the precise and timely

coupling of ATP-consuming and ATP-producing processes is a major question in cellular energetics (Bose *et al.* 2003; Dzeja & Terzic, 2003; Hochachka, 2003; Schlattner & Wallimann, 2004; Saks *et al.* 2005). Significant progress has been made in understanding molecular mechanisms of muscle contraction, cell motility, ion transport and the ATP synthesis machinery in mitochondria (Mitchell, 1979; Gordon *et al.* 2001; Nicholls & Ferguson, 2002; Berridge *et al.* 2003; Bianchi *et al.* 2004). However, the integration of different energetic units within the cell and the nature of signalling pathways that regulate their activities and provide linkage with ATP-utilizing and ATP-sensing components are a growing field of investigation. Here, we analyse paradoxes of cellular energetics and controversies existing at present in this

field, and highlight recent advances in cardiac energetics. We particularly underscore the emerging understanding of energy transfer and metabolic signalling networks that secure mechano-energetic coupling, and underlie heart physiology and the relationship between respiration rate and cardiac work in the classical Frank-Starling law.

Cardiac energetics: the Frank-Starling law and the 'stability paradox'

Under normoxia, cardiac energy metabolism relies on aerobic oxidation of fatty acids and carbohydrate substrates in mitochondria with the majority of ATP consumed by contractile machinery and ion pumps (Opie, 1998; Stanley *et al.* 2005; Taegtmeier *et al.* 2005). Therefore, mechanisms of intracellular feedback communication and regulation of mitochondrial respiration must be deciphered in order to understand the balanced coupling of ATP-producing and ATP-consuming processes *in vivo*. The phenomenological aspects of this regulation were initially recognized by the Starling laboratory in the period between 1914 and 1926, before ATP was discovered and long before the discovery of oxidative phosphorylation (Evans & Matsuoka, 1915; Starling & Visscher, 1926). The heart maintains normal blood circulation under a wide range of workloads, a function governed by the Frank-Starling law (Opie, 1998; Fuchs & Smith, 2001) originally described by Otto Frank (Frank, 1885) and Ernest Starling (Patterson *et al.* 1914; Starling & Visscher, 1926). This law states that cardiac performance increases with an increase in end-diastolic ventricular volume. In this way, the heart responds to increases in venous filling pressure (Patterson *et al.* 1914; Katz, 2002). However, this law has wider implications as it addresses the principles of cardiac mechano-energetic coupling (Opie, 1998). In their original experiments, Starling and co-workers used heart–lung preparations and measured the rate of oxygen consumption, which was taken as a measure of 'the total energy set free in the heart during its activity', an assumption recalling the XVIIIth century definition of biological oxidation by Lavoisier and Laplace (Evans & Matsuoka, 1915; Starling & Visscher, 1926; Vignais, 2005). As both work and respiration rates increased linearly with increase of left ventricular end-diastolic volume, the conclusion was made that 'any increase in the work demanded of the heart is met by a corresponding increase in the oxygen consumption and in the amount of chemical changes taking place' (Starling & Visscher, 1926). This is the metabolic underpinning of the Frank-Starling law. Within the century that followed Starling's work, detailed characteristics of the contraction cycle, energy consumption and heat production were determined as summarized elsewhere (Gibbs, 1978; Suga, 1990). One of the main parameters studied has been \dot{V}_{O_2} ,

the rate of oxygen consumption, described as a linear function of the pressure–volume area (PVA):

$$\dot{V}_{O_2} = A \times \text{PVA} + D$$

where, D is unloaded \dot{V}_{O_2} , PVA consists of the mechanical work within the contraction cycle and so-called 'potential energy' term, and A is a constant (which inversely describes the efficiency of the heart) (Suga, 1990). Another principal characteristic of heart energetics is that the linear increase in \dot{V}_{O_2} with workload is observed in the absence of measurable changes in the ATP and phosphocreatine (PCr) cellular content (Neely *et al.* 1972). This remarkable metabolic stability, also called 'metabolic homeostasis' (Balaban, 2002), justifies the use of \dot{V}_{O_2} as a parameter of energy consumption. Using the Frank-Starling mechanism and increasing the rate of left ventricular filling, Williamson and colleagues have shown that the rate of respiration in isolated working hearts can be changed by more than an order of magnitude, about 15–20 times, from an unloaded \dot{V}_{O_2} of around $8\text{--}12 \mu\text{mol min}^{-1} (\text{g dry wt})^{-1}$ to a maximal value of $170 \mu\text{mol min}^{-1} (\text{g dry wt})^{-1}$ (see Fig. 1; Williamson *et al.* 1976). This occurs under conditions of remarkable metabolic stability (Williamson *et al.* 1976). In the face of large changes in muscle work and respiration, this observed metabolic stability or homeostasis underlying Frank-Starling law is referred to as the 'stability paradox' (Hochachka, 2003).

The mitochondrial calcium cycle and respiration regulation paradox

Currently, central to the explanation of cellular mechanisms regulating mitochondrial respiration is the assumption of parallel activation of contraction and respiration by calcium (McCormack *et al.* 1990; Jouaville *et al.* 1999; Territo *et al.* 2001; Balaban, 2002; Bianchi *et al.* 2004; Brookes *et al.* 2004; Gunter *et al.* 2004).

Calcium liberated from local intracellular stores by calcium-induced calcium release mechanisms during excitation–contraction coupling activates the contraction cycle by binding to troponin C in the troponin–tropomyosin complex of thin filaments (Gordon *et al.* 2001). The mitochondrial calcium cycle, elucidation of which was started with pioneering works of Lehninger, Hunter and Carafoli (for a review see Carafoli, 2003), includes a calcium entry-and-export system that: (a) regulates calcium concentration in the mitochondrial matrix, where calcium is an important activator of the Krebs cycle dehydrogenases, thus increasing the capacity of oxidative phosphorylation (Denton *et al.* 1978; McCormack *et al.* 1990; Hansford & Zorov, 1998; Balaban, 2002); (b) allows active regulation of compartmentalized cytoplasmic calcium fluctuations and related signalling

(Berridge *et al.* 2003; Mackenzie *et al.* 2004); and (c) protects mitochondria from calcium overload (Hansford & Zorov, 1998; Bernardi, 1999; Rizzuto *et al.* 2000; Gunter *et al.* 2004; Jacobson & Duchen, 2004).

It is assumed that calcium signals match the demand for ATP in the cell to its production in mitochondria, and thus to the control of respiration (Jouaville *et al.* 1999; Territo *et al.* 2001; Balaban, 2002; Brini, 2003; Bianchi *et al.* 2004; Gunter *et al.* 2004; Brookes *et al.* 2004).

Although the 'calcium-only' hypothesis of respiration regulation has become popular, quantitative estimates of calcium effects in mitochondria are in conflict with the magnitude of changes in the respiratory rate *in vivo* (Fig. 1). Both experimental studies of calcium effects on the mitochondrial respiration *in vitro* (Territo *et al.* 2001) and mathematical modelling of mitochondrial metabolism (Cortassa *et al.* 2003) have shown that changes in calcium concentration can at maximum double respiration. This degree of activation of mitochondrial respiration is far too small to explain the energy flux changes of more than an order of magnitude that are observed in muscle cells *in vivo* (Fig. 1A). In cells with small fluctuations of energy fluxes, direct regulation of mitochondrial activity by calcium may be sufficient (Jouaville *et al.* 1999), but for excitable cells with high and rapidly fluctuating energy fluxes, such as the heart, skeletal muscle, brain and other cells, this is not the case. The decreased NADH/NAD ratio at higher workloads induced by increased ventricular filling, as observed by Williamson and colleagues (Williamson *et al.* 1976), is consistent with the metabolic feedback signalling regulating respiration *in vivo*. In the case of calcium-induced activation, an opposite effect is expected (Balaban, 2002): in this 'push' mechanism, the NADH/NAD ratio should be increased first, followed by an increase of respiration due to increased availability of this substrate to the respiratory chain.

Mitochondria are not only the main sites of cellular ATP generation, but also have a regulatory role in apoptosis and cell necrosis, thus making decisions on cell life and death (Bernardi, 1999; Rizzuto *et al.* 2000; Jacobson & Duchen, 2004; Brookes *et al.* 2004). Further increase of the mitochondrial calcium content above the optimal level would be extremely dangerous for the cell, since it results in inhibition of ATP synthesis (Holmuhamedov *et al.* 2001) and opening of the mitochondrial permeability transition pore (PTP) that would finally lead to cell death (Bernardi, 1999; Rizzuto *et al.* 2000; Brookes *et al.* 2004). This damaging effect of calcium is amplified by production of reactive oxygen species (ROS) within the respiratory chain (Brookes *et al.* 2004).

Most paradoxically, the 'calcium-only' hypothesis of respiration regulation contradicts conclusions made in cardiac physiology in the studies of the cellular basis of the Frank-Starling law. It was discovered by Hibberd

and Jewell and then confirmed by many others that the cellular mechanism behind the Frank-Starling law is the force-length relationship, a length-dependent activation of myofilaments due to increased sensitivity of the thin filaments to calcium at greater sarcomere length (Hibberd & Jewell, 1982; Allen & Kurihara, 1982; Landesberg, 1996; Landesberg & Sideman, 1999; Gordon *et al.* 2001;

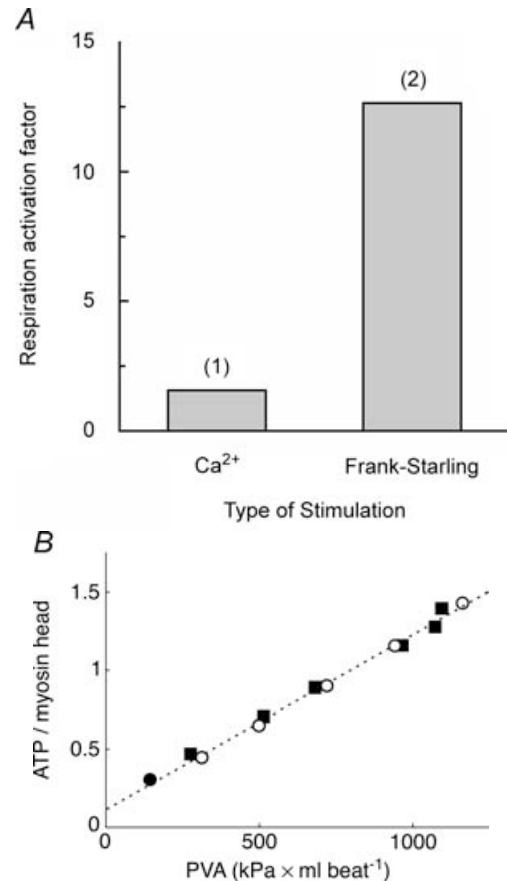


Figure 1. Conflict between the calcium hypothesis of regulation of mitochondrial oxidative phosphorylation *in vivo* and the high degree of activation of cardiac respiration by the Frank-Starling mechanism

A, the regulatory effects on the mitochondrial respiration rate of calcium in isolated mitochondria *in vitro* (1) (Territo *et al.* 2001), and that of changing left ventricle filling in isolated perfused rat hearts (2) (Williamson *et al.* 1976). The respiratory activation factor means the ratio of the maximal respiration rate to that in the absence of calcium (1) or in the arrested, non-contracting hearts (2). At zero calcium, the respiration rate of isolated mitochondria is already about 60% of V_{\max} observed at calcium concentrations higher than 600 nM (Territo *et al.* 2001). The difference between the degrees of activation of respiration by calcium and by Frank-Starling mechanism is of an order of magnitude. B, simulations demonstrating the influence of a mechanical loading protocol on the ATP consumption rate of muscle fibres in the left ventricle. ATP consumption by the left ventricle is shown as a function of pressure-volume area (PVA). The linear relationship (dashed line) between ATP consumption and PVA is the same for ejecting (●) and isovolumetric (□) contractions. (Adapted from Vendelin *et al.* 2002.)

Fuchs & Smith, 2001; Robinson *et al.* 2002; Katz, 2002). Intracellular calcium probes have revealed that practically no changes in the intracellular calcium transients accompany the stretching of sarcomeres sufficient to activate myofilaments (Kentish & Wrzosek, 1998; Shimizu *et al.* 2002). Only a slow increase of calcium transients after a length change was observed in the experiments of Allen & Kurihara (1982). However, it has clearly been shown in Suga's laboratory that the Frank-Starling mechanism does not affect intracellular calcium recirculation between sarcoplasm and sarcoplasmic reticulum (Mizuno *et al.* 2001). The number of strongly bound crossbridges and the force generated after calcium binding depend upon the length of sarcomeres (Gordon *et al.* 2001; Fuchs & Smith, 2001; Katz, 2002; Robinson *et al.* 2002). The mechanism of this length-dependent activation is complex and still under active study. It includes changes in myofilament lattice spacing with possible involvement of titin (Fukuda *et al.* 2001), resulting in a decrease of the distance between actin and myosin filaments and increased probability of crossbridge formation with an increase of sarcomere length (Millman, 1998; Fukuda *et al.* 2001), positive co-operativity of crossbridge binding to actin (Robinson *et al.* 2002) and an increase of the affinity of the troponin complex for calcium, induced by strong binding of crossbridges (Landesberg, 1996; Landesberg & Sideman, 1999; Gordon *et al.* 2001; Robinson *et al.* 2002). Due to these mechanisms, sarcomere stretch at submaximal calcium concentrations in cytoplasm results in an increase in the number of active crossbridges, and thus increased release of the products of ATP hydrolysis, first P_i during the power stroke, and then ADP (Landesberg & Sideman, 1999; Gordon *et al.* 2000, 2001). By this mechanism of sarcomere length-dependent activation of contraction, the steady-state rates of ATP consumption can change by an order of magnitude without any changes in the calcium cycle (Fig. 1B).

The question of how the rate of ATP production is increased by a factor of 15–20 to match the rates of ATP consumption in cardiomyocytes, while cardiac work is increased by a mechanism of length-dependent activation without the need of alteration of calcium signals, remains unresolved. If there are no changes in cytoplasmic calcium concentrations and/or in high-energy phosphate levels, which intracellular signal could prime mitochondria to increase the respiratory rate in a 'very energy-rich' intracellular environment (Colombini, 2004)?

The K_m (ADP) paradox and regulation of mitochondrial respiration

In many studies, cytoplasmic ADP concentration is calculated by assuming that creatine kinase (CK) is in full equilibrium in muscle cells *in vivo* (Meyer *et al.*

1984; Ingwall, 2002). The popularity of this approach lies in its simplicity to calculate global cellular ADP concentrations, found to be in the range of 50 to 100 μM , and then to deduce the value for the phosphorylation potential – the amount of free energy available in the $\text{ATP} + \text{ADP} + P_i$ system (Meyer *et al.* 1984; Gard *et al.* 1985; Unitt *et al.* 1992; Ingwall, 2002). However, since the rate of oxidative phosphorylation is regulated by the ADP availability to the adenine nucleotide translocator (ANT) and since the affinity of ANT for ADP is very high with an apparent $K_m(\text{ADP})$ between 5 and 20 μM even in the presence of ATP at physiological concentration (3–5 mM) (Vignais, 1976; Jacobus *et al.* 1982), respiration should always proceed, even at rest, with a rate $\geq 80\%$ of V_{\max} (between 120 and 130 μmol of $\text{O}_2 \text{ min}^{-1} (\text{g dry wt})^{-1}$). This would leave no margin for respiration regulation during workload changes, in conflict with experimental observations. Metabolic stability (or homeostasis) at vastly different workloads and respiration rates only confirms this conclusion: cytoplasmic ADP concentrations calculated from the supposed CK equilibrium are completely dissociated from the rates of respiration (From *et al.* 1986). Mathematical modelling has shown that the assumption of a CK equilibrium imposes an unnecessary limitation, masking the real mechanism of metabolic regulation of respiration (Saks & Aliev, 1996; see below). [There may still be some justification in calculating a virtual cytosolic phosphorylation potential from these ADP values, if this parameter is taken to show the possible average level or reserve of free energy potentially available in the $\text{ATP-ADP-}P_i$ system in the cell cytoplasm, where compartmentalized creatine kinase may approach the equilibrium state in cytoplasmic space in the diastolic phase of contraction (Aliev & Saks, 1997). However, for quantitative analysis of kinetic and energetic aspects of regulation of cellular respiration, this assumption is not appropriate. Instead, local ADP concentrations and phosphorylation potentials in different compartments calculated from mathematical models should be taken into consideration (Kinding *et al.* 2005a).]

The respiration rate *in vivo* seems to be controlled by processes and/or structures that can respond linearly to the ADP and P_i liberation within the contraction cycle. Such a regulatory mechanism also should be extremely efficient in removing cytosolic ADP. MgADP , as a close structural analogue of MgATP , is an efficient competitive inhibitor of ATPases, e.g. with a K_i close to 200 μM for filament sliding (Yamashita *et al.* 1994). In addition, MgADP induces the actomyosin–ADP complex which cooperatively promotes strong-binding crossbridges such as the rigor complexes and increases myofilament Ca^{2+} sensitivity, thus masking the length-dependent activation (Fukuda *et al.* 2000). The reversible ion pumps of the sarcoplasmic reticulum and sarcolemma are very sensitive to inhibition by MgADP

(de Meis & Inesi, 1982). Therefore, accumulation of MgADP in the vicinity of any ATPase slows down the contraction cycle (Yamashita *et al.* 1994; Sata *et al.* 1996) and impairs the Frank-Starling mechanism (Fukuda *et al.* 1998, 2000; Robinson *et al.* 2002). In conclusion, the cell needs to be protected from an excess in cytosolic free calcium, as well as from ADP, both potential regulators of mitochondrial respiration. The apparent affinity of oxidative phosphorylation for cytoplasmic ADP may be modified also by regulating the permeability of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (Saks *et al.* 1994; Lemeshko & Lemeshko, 2000; Colombini, 2004). In any case, local availability of ADP to ANT is a potential key regulator of respiration.

As will be outlined in the following, intracellular energy and metabolic signalling phosphotransfer networks within a defined cellular structural organization have the potential to both: (1) protect cells from the excess of cytosolic free calcium and ADP; and (2) regulate respiratory ATP production in close correspondence to ATP consumption.

Creatine kinase isoenzymes constitute a local and global energy transfer network

The major cellular energy transferring networks relies on isoenzymes of CK and the rapidly diffusable phosphocreatine (PCr) as a 'high-energy' intermediate (Fig. 2). CK catalyses the reversible transfer of 'high-energy' phosphoryl groups from PCr to ADP to generate ATP, or alternatively, from ATP to creatine (Cr) to generate PCr. CK acts as a very sensitive ADP sensor and the enzyme is able to remove ADP *in situ* by rephosphorylating it to ATP, tapping the large cellular PCr pool. Creatine kinase occurs in the form of multiple isoenzymes, with a cytosolic and a mitochondrial isoenzyme coexpressed in most tissues (Wallimann *et al.* 1992; Schlattner *et al.* 1998; Schlattner & Wallimann, 2004). These isoenzymes form an intricate high-energy phosphotransfer system not only for 'temporal buffering', but also for 'energy transport' or 'spatial energy-buffering' functions (Fig. 2*a–d*) (Bessman & Carpenter, 1985; Wallimann *et al.* 1992; Saks *et al.* 1994; Schlattner *et al.* 1998; Wyss & Kaddurah-Daouk, 2000; Saks *et al.* 2004; Schlattner & Wallimann, 2004), due to their subcellular localization at distinct sites and interaction with a number of cellular proteins, enzymes, biological membranes or organelles.

Mitochondrial CKs (MtCK) form octamers that are present (1) between the outer and inner mitochondrial membrane and (2) in the cristae space (Wallimann *et al.* 1992; Schlattner *et al.* 1998; Schlattner & Wallimann, 2005). The kinase catalyses the direct trans-phosphorylation of intramitochondrially produced ATP

and cytosolic creatine into ADP and PCr. ADP enters the matrix space to stimulate oxidative phosphorylation, giving rise to mitochondrial recycling of a specific pool of ATP and ADP, while PCr is the primary 'high-energy' phosphoryl compound that leaves mitochondria to enter the cytosol (Jacobus & Lehninger, 1973). The molecular basis for such directed metabolite flux is channelling between the large MtCK octamer (Schlattner *et al.* 1998; Schlattner & Wallimann, 2005) and two trans-membrane proteins, adenine nucleotide translocator (ANT), and mitochondrial porin or voltage-gated anion channel (VDAC) (Fig. 2). The MtCK-linked metabolite channelling is based on: (1) co-localization, (2) direct interactions, and (3) diffusion barriers (Vendelin *et al.* 2004*b*; Saks *et al.* 2005). MtCK tightly binds to cardiolipin, an acidic phospholipid that is specific for the mitochondrial inner membrane. Since ANT is situated in a cardiolipin patch, this leads to co-localization and metabolite channelling between both proteins in the cristae and intermembrane space (Schlattner *et al.* 2004). MtCK in the intermembrane space further interacts with outer membrane phospholipids and VDAC (Schlattner *et al.* 2001), thus virtually cross-linking the inner and outer membrane and contributing to the mitochondrial contact sites (Fig. 2, lower left). The limited permeability of VDAC and thus of the entire outer membrane creates a dynamic micro-compartmentation of metabolites in the intermembrane space (Gellerich *et al.* 1987) that contributes to MtCK-linked channelling and separate mitochondrial ATP and ADP pools (Andrienko *et al.* 2003).

In muscle, a significant fraction of the non-mitochondrial enzyme (MM-CK) is structurally and functionally associated or co-localized with: (1) different ion pumps in the plasma membrane, (2) the sarcomeric M-band of the myofibrils, and (3) the calcium pump of the sarcoplasmic reticulum (Rossi *et al.* 1990; Wallimann *et al.* 1992; Hornemann *et al.* 2000). Tight functional coupling of CK to ATPases, e.g. acto-myosin ATPase and ion pumps, such as the K^+/Na^+ -ATPase or the Ca^{2+} -ATPase, has the advantage: (1) that product inhibition of the ATPase by ADP and H^+ is avoided, since the latter two are both substrates of the CK reaction ($PCr^{2-} + MgADP^- + H^+ \leftrightarrow Cr + MgATP^{2-}$), and (2) that the high free energy of ATP hydrolysis (ΔG_{ATP}) at sites of ATP hydrolysis is preserved by keeping very high ATP/ADP ratios locally due to coupling of CK with those ATPases *in situ* and thus preventing energy dissipation caused by ATP transport and mixing with the bulk surroundings (Wallimann *et al.* 1992; Wyss & Kaddurah-Daouk, 2000; Dzeja & Terzic, 2003). There is increasing evidence for a restriction of ATP (and ADP) diffusion in the cell, particularly pronounced in the subsarcolemmal area (Abraham *et al.* 2002; Karpen & Rich, 2004; Selivanov *et al.* 2004; Vendelin *et al.* 2004*a*). These restrictions are bypassed by the CK–PCr system

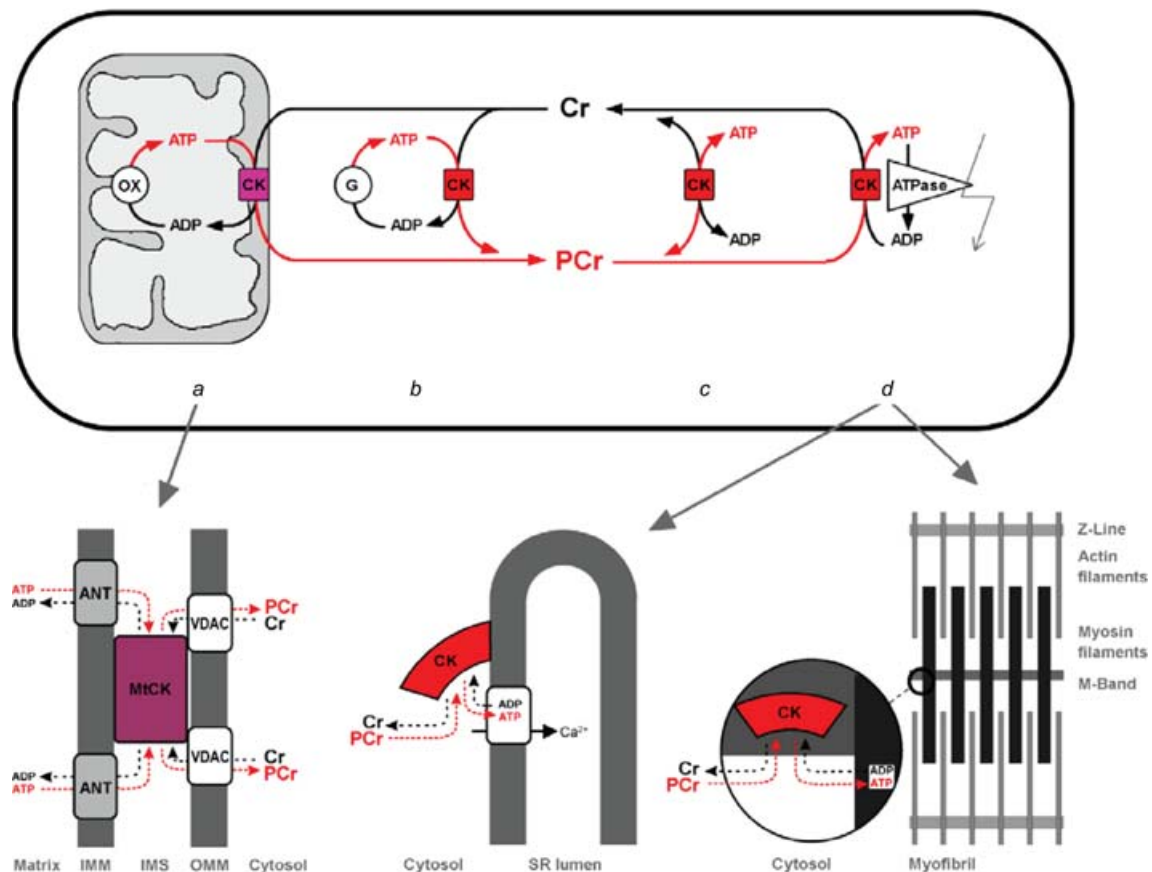


Figure 2. The creatine kinase–phosphocreatine energy transfer system and selected CK microdomains

Overview (top): isoenzymes of creatine kinase (CK) are found in different compartments like mitochondria (a) and cytosol (b–d), in soluble form (c) or associated to a different degree with ATP delivering (a and b) or -consuming processes (d). A large cytosolic phosphocreatine (PCr) pool of up to 30 mM is built up by CK using creatine (Cr) and ATP preferentially from oxidative phosphorylation (OX), e.g. in heart (a) or from glycolysis (G), e.g. in fast-twitch glycolytic muscle (b). PCr is then used to buffer global (c) and local (d) ATP/ADP ratios. In cells that are polarized and/or have very high or localized ATP consumption, these CK isoenzymes, together with easily diffusible PCr, also maintain an energy shuttle between ATP-providing or -consuming processes (a and d) (Wallimann *et al.* 1992). Metabolite channelling occurs where CK is associated with ATP-providing or -consuming transporters, pumps or enzymes (a, b and d) (Schlatterner & Wallimann, 2004). Substrate and product fluxes between MtCK and the associated proteins are depicted as arrows. MtCK metabolite channelling in mitochondrial microcompartments (lower left): octameric MtCK is found in two locations: (i) in the mitochondrial intermembrane space (IMS), where it associates in the so-called contact sites with the adenine nucleotide translocator (ANT) as well as the voltage-dependent anion channel (VDAC, or porin), and (ii) in the cristae where it associates with ANT only (not shown). In contact sites, octameric MtCK binds simultaneously to inner (IM) and outer mitochondrial membranes (OM), due to the identical top and bottom faces of the octamer. The binding partner in the IM is the 2-fold negatively charged cardiolipin, which allows a functional interaction with adenine nucleotide translocator (ANT) that is situated in cardiolipin membrane patches (Schlatterner *et al.* 2004). In the OM, MtCK interacts with other acidic phospholipids and, in a calcium-dependent manner, directly with the regulated pore-forming VDAC (Schlatterner *et al.* 2001). In contact sites, the metabolite channelling allows for a constant supply of substrates and removal of products at the active sites of MtCK. By preventing the dissipation of mitochondrially generated ATP into the cytosol, its energy content is directly transferred by MtCK to Cr, to give PCr, a metabolically inert compound. PCr then leaves via VDAC into the cytosol, where it is available to CKs at the various locations for *in situ* regeneration of ATP. *Vice versa*, intramitochondrial regeneration of ADP stimulates oxidative phosphorylation (OX) (Schlatterner *et al.* 1998). Microcompartments of cytosolic MM-CK with the SR Ca^{2+} -ATPase pump (lower middle): a fraction of total cytosolic muscle MM-CK is bound to the SR where it maintains a high local ATP/ADP ratio and is functionally coupled to the Ca^{2+} -ATPase pump (Rossi *et al.* 1990). ATP provided to the pump is preferentially delivered from PCr by bound CK and the apparent K_m for ATP for the calcium pump is lowered by this functional interaction. The energetically demanding Ca^{2+} pump operates close to thermodynamic equilibrium and thus, for efficient sequestration of Ca^{2+} , depends on a high local ATP/ADP ratio. Microcompartments of MM-CK in the sarcomeric M-band of the myofibrillar contractile apparatus (lower right): a further fraction of cytosolic muscle MM-CK is bound to the sarcomeric M-band of myofibrils, sufficient in activity to fully regenerate the ATP hydrolysed by the

due to the attachment of the MM-CK isoenzyme to the sarcolemma. It was shown directly that the CK system maintains a close functional coupling between mitochondria and ion channels in sarcolemma (Sasaki *et al.* 2001; Abraham *et al.* 2002).

Thus, the CK-PCr circuit represents an efficient regulator of energy flux and uses metabolite channelling as a fine-tuning device of local ATP levels. The significance of such a regulated channelling circuit operating at the high total PCr and Cr pools lies in its high sensitivity towards ADP that it prevents, especially in excitable cells, the accumulation of ADP, and consequently AMP through the adenylate kinase reaction (Dzeja *et al.* 1998; Dzeja & Terzic, 2005), unless severe stress, such as hypoxia or ischaemia is imposed. In the latter case, AMP-activated protein kinase (AMPK) and other AMP-sensitive components would be activated by free AMP initiating signalling cascades that would turn on compensatory mechanisms for increasing energy supply and reducing energy consumption (Pucar *et al.* 2004; Kahn *et al.* 2005).

Integrated phosphotransfer and signalling networks in regulation of cellular energy homeostasis

Besides the CK-PCr system described above, additional and complementary phosphotransfer networks comprising adenylate kinase isoforms and glycolytic enzymes as well as the energy sensor AMP-activated protein kinase (AMPK), work hand in hand with CK (Dzeja & Terzic, 2003; Neumann *et al.* 2003; Kahn *et al.* 2005). One of the major outcomes resulting from CK and AK1 knockout studies was the discovery that glycolytic enzymes have the ability to provide a network capacity for transferring and distributing high-energy phosphoryls (Dzeja *et al.* 1998, 2004; de Groof *et al.* 2001; Pucar *et al.* 2002; Janssen *et al.* 2003a). This new 'spatial' look at glycolytic metabolism together with the robust phosphotransfer capacity, especially by the GAPDH-PGK (glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase) couple (From *et al.* 1986; Kingsley-Hickman *et al.* 1987), renders glycolysis an equal partner in the cellular energy distribution network along with CK and AK phosphotransfer pathways (Dzeja & Terzic, 2003; Dzeja *et al.* 2004). The function of the newly discovered adaptor protein DRAL/FHL-2, which is involved in anchoring CK, adenylate kinase and glycolytic enzymes to sites of high-energy consumption (Lange *et al.* 2002), further highlights the significance of maintaining the structural integrity of intracellular phosphotransfer networks for matching cellular energy needs.

Further insights and key support for the current understanding of metabolic signalling networks in their full complexity have come with the development of new methodologies (Dzeja *et al.* 1998). High-energy phosphoryl fluxes through CK, adenylate kinase and glycolytic phosphotransfer, captured with ^{18}O -assisted ^{31}P NMR, tightly correlate with the performance of the myocardium under various conditions of stress load (Pucar *et al.* 2004) (Fig. 3), implicating phosphotransfer reactions as indispensable routes that direct flow of high-energy phosphoryls between cellular ATPases and the ATP production machinery in mitochondria. Another unique feature of the ^{18}O methodology is that it allows direct monitoring of intracellular energetic communication by following the $[\text{O}^{18}\text{P}]/[\text{O}^{18}\text{O}]\gamma\text{-ATP}$ labelling ratio, a novel parameter of energetic signalling between cellular compartments (Pucar *et al.* 2001). Changes in this parameter indicate that communication between cellular ATPases and ATPsynthases is reduced by myocardial ischaemia, heart failure and transgenic phosphotransfer enzyme deficiencies (Pucar *et al.* 2001; Janssen *et al.* 2003a,b; Dzeja *et al.* 2004). This new methodology allows quantitative evaluation of cellular ATP turnover and the distribution of energy fluxes between different phosphotransfer pathways *in vivo*. In the experiments with Langendorff-perfused hearts shown in Fig. 3, the creatine kinase flux increased linearly with cardiac performance (evaluated as the rate-pressure product), and reached the value close to $300 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ with the heart performing at about $30\,000 \text{ mmHg min}^{-1}$, corresponding to a rate of oxygen consumption of about $40 \mu\text{mol O}_2 \text{ min}^{-1} (\text{g dry weight})^{-1}$ (Dos Santos *et al.* 2000). Taking into account the CK flux in the equilibrium state (in arrested heart) (Fig. 3), protein content of tissue of about $150 \text{ mg (g wet weight)}^{-1}$, and the wet weight/dry weight ratio of 5, these data show the PCr/O₂ ratio close to 5. This supports the notion that the CK pathway carries the major part of energy flux out of mitochondria to the ATPases under normal physiological conditions in heart cells as described above (Fig. 2), in agreement with mathematical modelling of the compartmentalized energy transfer (Aliev & Saks, 1997). About 10–15% of cellular high-energy phosphoryls can be carried by the combined AK-glycolytic systems, whose contribution increases with muscle contraction and in failing hearts (Dzeja *et al.* 1998, 1999; Pucar *et al.* 2001). Such flux distribution between CK, AK and glycolytic systems is based on the assumption of parallel phosphotransfer pathways. However, in the cellular environment, these pathways are closely co-localized and interconnected

actomyosin ATPase at maximal contraction velocity. MM-CK is specifically anchored in an isoenzyme-specific manner at the M-band via two 'lysine charge clamps' that facilitate the binding of the enzyme to two structural proteins, M-protein, as well as myomesin, at this sarcomeric location (Hornemann *et al.* 2000).

allowing high-energy phosphoryls to flow from one system to another (Kupriyanov *et al.* 1984; Bessman & Carpenter, 1985; Wallimann *et al.* 1992; Janssen *et al.* 2000; Dzeja & Terzic, 2003; Dzeja *et al.* 2004). Indeed, labelling studies indicate that about 1/3 of phosphoryls in PCr come from the adenine nucleotide β -phosphoryls through the AK catalysis (Bessman & Carpenter, 1985). Also, there is an intimate interaction and complementation between AK, CK and glycolytic phosphotransfers at myofibrillar and mitochondrial sites (Dzeja *et al.* 1998; de Groof *et al.* 2001; Neumann *et al.* 2003; Dzeja & Terzic, 2005). The role and contribution of individual phosphotransfer enzymes also depend on the species, tissue, developmental stage or physiological state, underscoring the plasticity of the cellular energetic system in governing metabolic homeo-

stasis (Dzeja *et al.* 2002; Dzeja & Terzic, 2005). The linear relationship between the creatine kinase flux and the workload (and thus the respiration rate) is also consistent with its measurements by the ^{31}P -NMR saturation transfer method (Kupriyanov *et al.* 1984; Bittl & Ingwall, 1985). The latter method is, however, rather 'global' and the results of its use may sometimes be misinterpreted (Wallimann, 1996). Further complicating global flux analysis of the system, the directions of CK reaction fluxes are opposite at subcellular sites of energy utilization, where bound CK regenerates ATP, and at mitochondria, where CK produces PCr. Also, up to 35% of total CK flux, most probably corresponding to compartmentally bound CK, is 'NMR-invisible' (van Deursen *et al.* 1994; Wallimann, 1996). However, by a new method of NMR CK flux

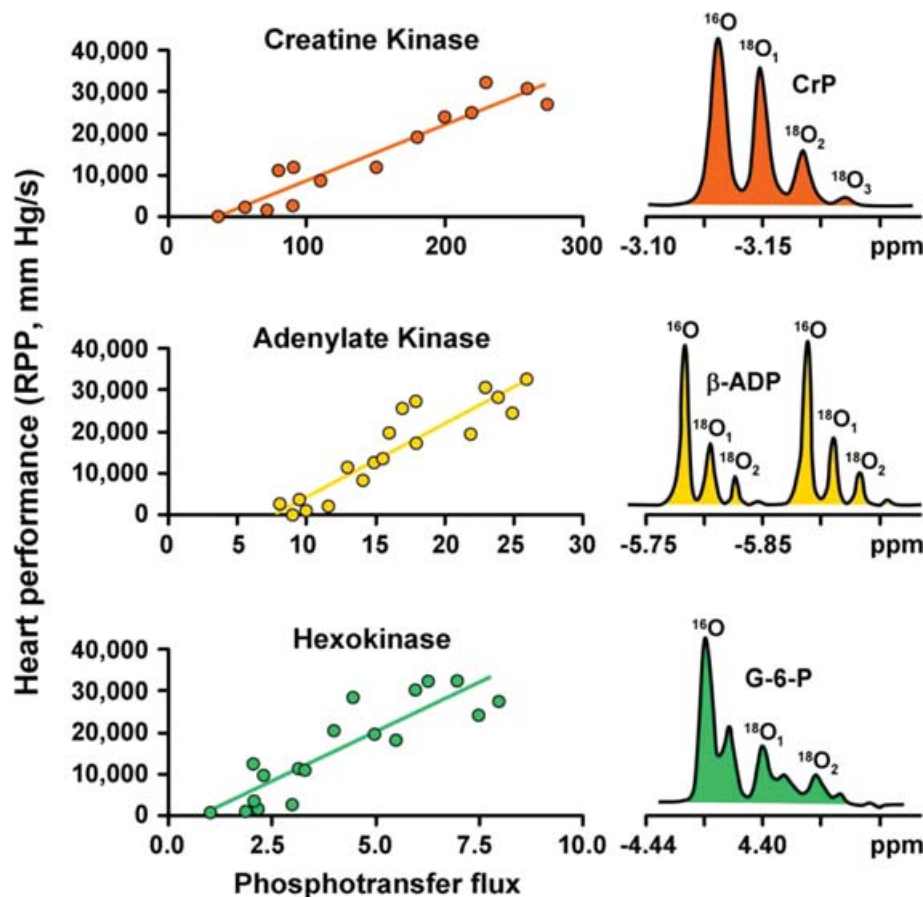


Figure 3. Phosphotransfer networks mediate coupling between heart functional activity and ATP generation

Processing of high-energy phosphoryls through creatine kinase (CK), adenylate kinase (AK) and glycolytic (hexokinase) systems correlates linearly with heart functional activity (left panels). Phosphotransfer flux measurements obtained from ^{18}O -induced shift in ^{31}P -NMR spectra of phosphocreatine (CrP), β -ADP and glucose-6-phosphate (G-6-P) (right panels) (Pucar *et al.* 2001). Appearance of $^{18}\text{O}_1$, $^{18}\text{O}_2$ and $^{18}\text{O}_3$ peaks in corresponding phosphoryl-containing metabolites indicates phosphotransfer flux through the CK, AK and hexokinase steps in the glycolytic system (Dzeja *et al.* 2004). Control hearts and contractile function modulated by ischaemia-reperfusion are included in calculations (Pucar *et al.* 2001). RPP, rate pressure product; phosphotransfer flux is expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ of corresponding metabolite.

analysis (Joubert *et al.* 2001), it was possible to demonstrate multicompartment CK fluxes that are consistent with the PCr–CK shuttle (Joubert *et al.* 2004).

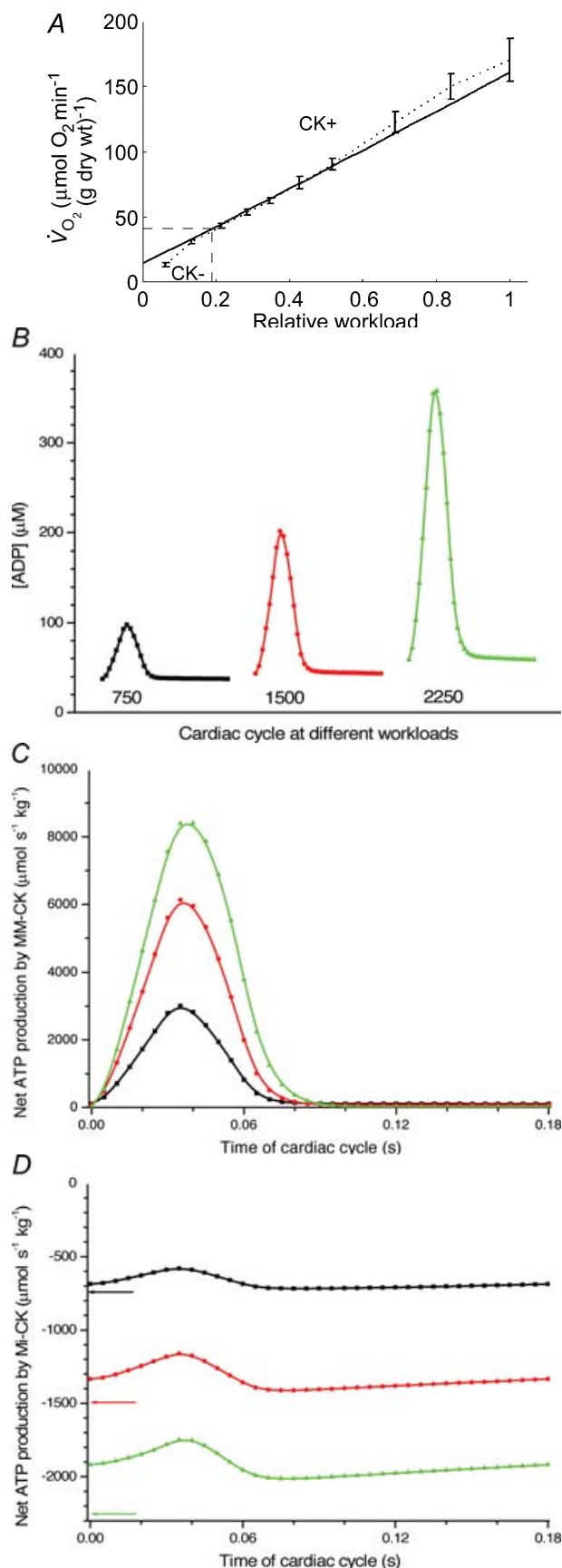
The ^{18}O -assisted ^{31}P NMR and mass spectrometry have less complications compared with the ^{31}P NMR saturation or inversion spin transfer methods (Dzeja *et al.* 1998, 2004; Pucar *et al.* 2001), allowing quantitative studies of *in vivo* distribution of high-energy phosphoryl fluxes through different metabolic pathways (Janssen *et al.* 2000). The high values of the CK fluxes and their linear relationship with workload measured by this method are consistent with a central role of the CK system in ergo-mechanical coupling underlying the heart's Frank-Starling law.

Phosphotransfer systems in metabolic feedback regulation

Due to a high degree of structural organization and diffusional restrictions, intracellular energetic signalling employs distinct mechanisms of communication, ranging from substrate channelling, reaction-diffusion and ligand conduction, depending on the specific compartment, molecular organization and topological arrangement of enzymatic networks (Bessman & Carpenter, 1985; Saks *et al.* 1994, 2004; Dzeja & Terzic, 2003, 2005; Schlattner & Wallimann, 2004). While the spatial heterogeneity and directionality of enzyme-catalysed processes are not possible in well mixed conditions *in vitro*, this becomes a vital entity in highly organized living matter (Mitchell, 1979). In such organized enzymatic networks a wide range of metabolic flux changes can be induced and propagated at relatively stable levels of intermediates (Dzeja *et al.* 1998; Vance *et al.* 2002). The studies, initiated by the discovery of organized enzyme clusters, so-called metabolons by Srere some 30 years ago (for review see Srere, 2000), have significantly contributed to the development of a new, holistic approach to cell metabolism and regulation, for which the term 'molecular system biology' has been coined (Kitano, 2002; Noble, 2002). Cluster organization and the high rate of unidirectional phosphoryl exchange in phosphotransfer systems promotes ligand conduction and signal communication at cellular distances providing enhanced thermodynamic efficiency (Mitchell, 1979; Saks *et al.* 1994, 2004; Dzeja *et al.* 1998). Indeed, the surrounding of mitochondria is properly ordered in muscle cells *in vivo* to avoid the potential chaos of macromolecular crowding, and mitochondria are arranged in a very regular pattern (Vendelin *et al.* 2005), forming intracellular energetic units (ICEUs), functional complexes with adjacent MgATPases of sarcoplasmic reticulum and myofibrils (Saks *et al.* 2001; Seppet *et al.* 2001; Vendelin *et al.* 2004a). ICEUs are analogous to calcium release units (CRUs), structurally organized sites of Ca^{2+} microdomains (Ca^{2+} sparks) which form

a discrete, stochastic system of intracellular calcium signalling in cardiac cells (Wang *et al.* 2004). The structural organization of ICEUs results in local confinement of adenine nucleotides and Cr–PCr couple in discrete dynamic energetic circuits between actomyosin ATPases and mitochondrial ATPsynthases (Dzeja *et al.* 1998; Saks *et al.* 2004). Similar discrete microdomains in cardiomyocytes have been shown for cAMP in the range of approximately $1\ \mu\text{m}$ and with high local concentrations (Zaccolo & Pozzan, 2002). Assuming that all units function in a synchronized manner, it would be sufficient to describe quantitatively the regulation of mitochondrial function within one such ICEU unit (Aliev & Saks, 1997; Vendelin *et al.* 2000). Indeed, it has been shown by Aon *et al.* (2003) that depolarization by a two-photon laser of a small number of mitochondria in a very limited area of the cell did not change the polarization of mitochondria in other parts of the cell. However, the mitochondrial polarization state did begin to oscillate in a synchronized manner after a substantial delay, triggered by local release of reactive oxygen species which in turn generated cyclic changes in the action potential (Aon *et al.* 2003). This is consistent with multiple studies showing that in cardiac and many other type of cells, mitochondria are morphologically and functionally heterogeneous (Zorov *et al.* 2000, 2004; Collins *et al.* 2002; Kuznetsov *et al.* 2004), resulting in depolarization of separate mitochondria ('black hole' phenomenon) in pathological states due to reactive oxygen species (ROS)-induced ROS release (Zorov *et al.* 2000) or mitochondrial permeability transition induced by calcium overload (Kuznetsov *et al.* 2004; Zorov *et al.* 2004). It is still an open question and an exciting task to discover which genes and proteins are responsible for the very regular arrangement of mitochondria in heart cells. The proteins controlling the fission and fusion of mitochondria are expressed in muscle cells, but these processes are strongly restricted by a highly organized cell structure and cytoskeletal arrangement (Yaffe, 1999; Rube & van der Blik, 2004). Further studies should address how the regular arrangement of mitochondria into ICEUs in muscle cells is established from chaotic or stochastic mitochondrial dynamics during muscle development.

There are already several interesting mathematical models of mitochondrial function and cardiac energy metabolism, reviewed recently by Jafri *et al.* (2001). A quantitative analysis of respiration regulation in the ICEUs under the physiological conditions of the Frank-Starling law can be achieved with a mathematical model of compartmentalized energy transfer sustained by the coupled CK system (available at: http://cens.ioc.ee/~markov/etransfer/current_model.pdf) (Fig. 4A). This model shows that ADP released from crossbridges is rapidly rephosphorylated by myofibrillar CK, producing small but opposite changes in Cr and PCr concentrations (Vendelin *et al.* 2000). This results



in cyclic changes in the concentration of ADP in the core of myofibrils in ICEUs (Fig. 4B) in a micro-compartment containing myofibrillar-bound MMCK, where ADP is first produced by actomyosin–MgATPase during the contraction cycle of crossbridges, and then rephosphorylated by CK due to a non-equilibrium state of the CK reaction (Vendelin *et al.* 2000). Thus, cyclic changes in the local ADP production are immediately displacing the myofibrillar MM-CK reaction in the direction of local ATP regeneration (Fig. 4C). The amplitude of displacement of CK from equilibrium (as well as cyclic changes of ADP) is proportionally increased with workload (Fig. 4B and C) (Vendelin *et al.* 2000). Without CK, the changes of local ADP concentrations in these microcompartments will be much more dramatic (Aliev & Saks, 1997; Saks *et al.* 2005). If myoplasmic CK is structurally organized and bound to the cytoskeleton, these cyclic changes may be channelled to mitochondria by a mechanism of so-called ‘vectorial ligand conduction’ – spatially directed group translocation down gradients of group potential (Mitchell, 1979; Saks *et al.* 1994, 2005;

Figure 4. Mathematical modelling of the regulation of mitochondrial respiration in cardiac cells *in vivo* under physiological conditions controlled by the Frank-Starling mechanism

A, computed (continuous line) and experimental (points with standard deviations, from Williamson *et al.* (1976) oxygen consumption rates of working cardiac muscle. Relative workload is the fraction of maximal workload applied, in computations this is the rate of ATP hydrolysis by actomyosin–MgATPase. CK+ shows the calculations for active creatine kinase system, CK- shows the calculations for the cells with inactivated creatine kinases (results shown within the dashed square). B, ADP profiles in the myoplasmic space in the core of myofibrils of ICEUs over cardiac cycle at different workloads (in the presence of CK). The model includes the diffusion restriction of ADP through mitochondrial outer membrane (Vendelin *et al.* 2000). Profiles correspond to the workloads equivalent to 750 (black), 1500 (red) and 2250 (green) $\mu\text{mol ATP s}^{-1} \text{ kg}^{-1}$. Reproduced from Vendelin *et al.* (2000) with permission. C, workload dependence of the dynamics of net ATP production by MM-CK in myofibrils. The reaction rates for workloads of 750 (black), 1500 (red) and 2250 (green) $\mu\text{mol ATP s}^{-1} \text{ kg}^{-1}$ are shown. Net rate of the reaction means displacement from the equilibrium position of the creatine kinase reaction (observed in diastolic phase) as the result of local ADP production in the contraction cycle (Fig. 4B). Courtesy of M. Aliev. D, workload dependence of the dynamics of net PCr production by mito-CK. Workload values are indicated by the arrows of respective colour. Complete model of Dos Santos *et al.* (2000), used in simulations in Saks *et al.* (2005). Mitochondrial membrane potential $\Delta\Psi = -160 \text{ mV}$, V_{max} of ATP-synthase = $4269.3 \mu\text{mol ATP s}^{-1} \text{ kg}^{-1}$. PCr export by mitochondria is 90.9, 89.1 and 85.8% of total mitochondrial energy export for workloads of 750, 1500 and 2250 $\mu\text{mol ATP s}^{-1} \text{ kg}^{-1}$, respectively. Due to the functional coupling between ANT and MtCK (Fig. 2), the reaction runs always out of equilibrium in the direction of PCr synthesis (Vendelin *et al.* 2004b), the steady-state values of the rates of this coupled reactions are increased by increasing the workload (cyclic changes of MgATP and MMCK reactions in myofibrils) and metabolic signalling of these changes to mitochondria by the creatine kinase–phosphocreatine energy transfer system (see Fig. 2 and the text). Courtesy of M. Aliev.

Dzeja & Terzic, 2005). The stimulatory effect of these CK ligands (Cr and/or ADP) on mitochondrial respiration is amplified by the functional coupling between MtCK and ANT (Schlattner *et al.* 1998; Saks *et al.* 2004, 2005; Schlattner & Wallimann, 2004). Because of this coupling, MtCK always catalyses the steady-state unidirectional PCr and ADP production from mitochondrial ATP and cytoplasmic creatine (Aliev & Saks, 1997; Vendelin *et al.* 2004b) (Fig. 4D). Within the whole contraction cycle these coupled reactions are in the steady state (Fig. 4D), in which the rates of ADP and ATP cycling and thus the respiration in mitochondria coupled to the PCr production (see Fig. 2) are increased with elevation of the workload (Fig. 4D). Accompanying changes in Cr, PCr and total ATP are within the experimental errors of their detection, thus giving an overall impression of metabolic stability (Vendelin *et al.* 2000; Wallimann, 1996). In this regard, CK, adenylate kinase and other phosphotransfer isoenzymes in different intracellular compartments are 'pushed' or 'pulled' from the equilibrium in opposite directions, depending on the activity of an associated process which drives steady-state high-energy phosphoryl flux (Saks *et al.* 1994; Dzeja *et al.* 1998).

The complex organization of this regulatory network has, paradoxically, a very simple purpose: to respect the conservation equation of ATP synthesis: $\text{ADP}^{3-} + \text{P}_i^{2-} + \text{H}^+ \leftrightarrow \text{ATP}^{4-}$ (Garlid, 2001). Under conditions of metabolic stability, an increase in the rate of ATP synthesis and of the coupled respiration (oxidative phosphorylation) is possible only if the supply of ADP and P_i to mitochondria is increased, making both metabolites good candidates for metabolic feedback signals controlling respiration. ADP is supplied by the metabolic signalling networks described above, to overcome the possible restriction of its diffusion and to avoid the inhibition of MgATPases. Additionally, the CK reaction, if running in the direction of ATP regeneration, compensates for pH changes due to MgATPase hydrolysis (Wallimann *et al.* 1992). Application of the principles of Metabolic Control Analysis, a universal methodological approach to study regulation of multienzyme systems (Westerhoff & VanDam, 1987; Bose *et al.* 2003), to the mathematical model showed that in parallel to the cyclic changes in ADP, at least at low workloads, P_i flux to mitochondria plays an important regulatory role (Saks *et al.* 2000). This conclusion was confirmed experimentally (Bose *et al.* 2003). Thus, the feedback metabolic signal has a complex nature, several of its components such as P_i , ADP and Cr may act in parallel, and their relative contribution changes with workload (Saks *et al.* 2000; Dzeja & Terzic, 2003). In any case, as a response to sarcomere stretch and crossbridge cycling, this complex signal leads to increases in the respiration rate, coupled mitochondrial PCr production, and energy flux via the CK–PCr system,

while also maintaining metabolic stability (homeostasis) at such an elevated workload.

'Metabolic pacing': synchronization of electrical and mechanical activities with energy supply

It is equally important for the normal functioning of cardiac cells that the isoenzymes of adenylate kinase and CK regulate the activity of diverse metabolic sensors, such as the ATP-sensitive sarcolemmal K^+ (K_{ATP}) channel, which opening reduces the duration of the action potential and thus the entry of calcium into the cell (Noma & Shibasaki, 1985; Weiss & Venkatesh, 1993; Dzeja *et al.* 1998; Carrasco *et al.* 2001; Abraham *et al.* 2002; Neumann *et al.* 2003; Hodgson *et al.* 2003; Pucar *et al.* 2004). In the heart, the K_{ATP} channel metabolic sensor function is manifested in a feedback response to cellular energetic signals adjusting membrane potential-dependent functions to harmonize myocardial functional activity (Weiss & Venkatesh, 1993; Sasaki *et al.* 2001; Alekseev *et al.* 2005). Genetic deficiency, pharmacological inhibition or altered metabolic sensing of K_{ATP} channels are associated with disturbed intracellular Ca^{2+} handling, excitation–contraction coupling and maladaptive stress response (Rajashree *et al.* 2002; Hodgson *et al.* 2003; Kane *et al.* 2004). In humans, mutations in K_{ATP} channel sub-units producing, defective metabolic sensing, increase susceptibility to dilated cardiomyopathy (Bienengraeber *et al.* 2004).

Basic principles of phosphotransfer-mediated metabolic signalling to metabolic sensors at the sarcolemma are presented in Fig. 5. The premembrane area, where diffusion of molecules is restricted due to an unstirred layer of structured water and molecular crowding (Abraham *et al.* 2002; Selivanov *et al.* 2004; Saks *et al.* 2005), represents the 'sensing zone' of a metabolic sensor, where intimate changes in nucleotide ratios are sensed and transduced into appropriate cellular responses (Alekseev *et al.* 2005; Dzeja & Terzic, 1998; Selivanov *et al.* 2004). Since cellular and even submembrane ATP concentrations are higher than required for half-maximal channel inhibition (between 10 and 100 μM) (Noma & Shibasaki, 1985; Abraham *et al.* 2002; Selivanov *et al.* 2004), production of ADP by AK or membrane ATPases and ADP scavenging by CK and PK reactions are critical for channel activity. It may be assumed that dynamic interaction between creatine kinase (CK), adenylate kinase (AK) and glycolytic enzymes (represented as pyruvate kinase, PK) phosphotransfer relays determines the behaviour of metabolic sensors such as the K_{ATP} channel – and subsequent cellular responses, such as excitability, hormone secretion, intracellular calcium homeostasis and vascular tone (Carrasco *et al.* 2001;

Sasaki *et al.* 2001; Abraham *et al.* 2002; Dzeja & Terzic, 2003; Alekseev *et al.* 2005; Dhar-Chowdhury *et al.* 2005; Kane *et al.* 2005). These phosphotransfer relays communicate metabolic signals originating in mitochondria or at cellular ATPases to metabolic sensors conveying information about 'high' or 'low' cellular energy, oxygen supply or hormonal states (Dzeja & Terzic, 1998; Tarasov *et al.* 2004). CK and AK deficiencies or their altered activity ratio compromise metabolic signalling to K_{ATP} channels (Carrasco *et al.* 2001; Abraham *et al.* 2002). Decreases in CK activity, such as in CK knockouts or failing hearts, can be partially compensated by the increase in glycolytic phosphotransfer to alleviate cardiomyocyte electrical instability (Dzeja *et al.* 1999; Abraham *et al.* 2002; Hodgson *et al.* 2003). Thus, phosphotransfer-governed nucleotide exchange and metabolic sensing is essential in processing cellular information and sustaining energy, ionic and hormonal homeostasis and in generating synchronized electrical and metabolic pacing ensuring cellular energetic homeostasis (Fig. 6). As such, during the cardiac cycle, an electrical impulse-induced action potential and membrane depolarization causes Ca^{2+} influx through L-type Ca^{2+} channels in the sarcolemma and T-tubules triggering Ca^{2+} release from intracellular stores in the sarcoplasmic reticulum (SR) through ryanodine receptor-channels (RyR) and cardiomyocyte contraction (Bers, 2002; Foell *et al.* 2004). ATP consumed during myofibrillar contraction and Ca^{2+} pumping is rapidly replenished by CK, AK and glycolytic phosphotransfer relays maintaining optimal local ATP/ADP ratios and

free energy of ATP hydrolysis (Wallimann *et al.* 1992; Saks *et al.* 1994; Dzeja & Terzic, 2003; Ingwall, 2004). Simultaneously these systems generate and translate feedback signals to stimulate ATP production in mitochondria and to convey information to energy demand sensors, such as K_{ATP} channels (and also AMPK) (Dzeja & Terzic, 1998; Neumann *et al.* 2003; Dzeja *et al.* 2004; Schlattner & Wallimann, 2004; Saks *et al.* 2004). The amount of Ca^{2+} entering a cell is controlled by the action potential duration regulated by K_{ATP} channels located in sarcolemma and T-tubules (Sasaki *et al.* 2001; Kane *et al.* 2004; Alekseev *et al.* 2005). Conversely, the activity of this metabolic sensor is regulated by phosphotransfer reaction and Ca^{2+} feedbacks (Weiss & Venkatesh, 1993; Dzeja & Terzic, 1998; Wilson *et al.* 2000; Selivanov *et al.* 2004). Ca^{2+} entering the cell or released from intracellular stores activates substrate transport (recruitment of glucose transporters), dehydrogenase (DH) activity (such as pyruvate dehydrogenase, an entry point into the mitochondrial Krebs cycle), and glycogenolysis to meet incoming energy needs (Hansford & Zorov, 1998; Balaban, 2002; Cortassa *et al.* 2003). Simultaneous recruitment of different steps in the cellular energetic system and ligand conduction type handling of substrates in phosphotransfer systems increase metabolic flux without apparent changes in substrate concentrations (Mitchell, 1979; Saks *et al.* 1994; Dzeja *et al.* 1998).

Thus, phosphotransfer-mediated metabolic feedback and Ca^{2+} signalling are important components in regulated signal transmission and coordinated

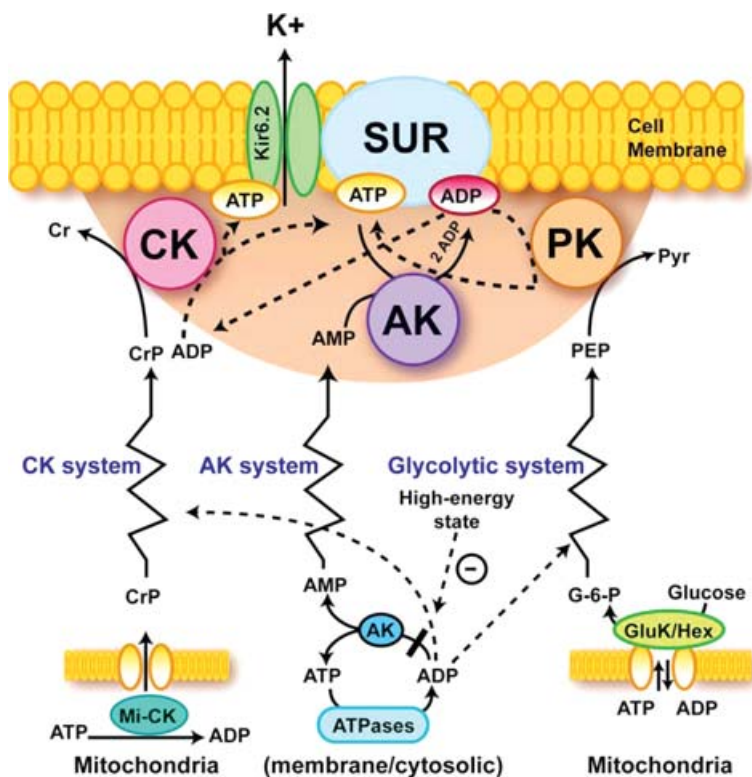


Figure 5. A paradigm of phosphotransfer-mediated energetic signalling: coupling cellular metabolic and electrical activities

Dynamic interaction between creatine kinase (CK), adenylate kinase (AK) and glycolytic (represented by pyruvate kinase, PK) phosphotransfer relays determines a prototypic metabolic sensor – K_{ATP} channel behaviour and subsequent cellular responses, such as excitability, hormone secretion, intracellular calcium homeostasis and vascular tone. The shadowed area represents a metabolic sensor 'sensing zone', where intimate local changes in nucleotide ratios are sensed and transduced into an appropriate cellular response. Phosphotransfer circuits connect the 'sensing zone' with cellular processes. Dashed lines indicate pathways signalling the high-energy state, while continuous lines represent low-energy state signal transmission. Kir6.2, potassium channel subunit; SUR, sulfonylurea receptor; GluK/Hex, glucokinase and hexokinase. Used with permission from Am. J. Physiol 2005.

dehydrogenase activation producing an optimal mitochondrial response to increased cellular energy demands.

Metabolic channelling is needed for a wide range of workloads and protects the cell from deleterious effects of calcium overload

The physiological mechanism of respiration regulation described above has the important advantage of ensuring effective control of free energy conversion across the whole physiological range of workloads, without requiring a severe increase in cytoplasmic calcium concentration. It thus avoids any danger of mitochondrial calcium overload that would open the mitochondrial permeability transition pore and thus lead to cell death. Without such a mechanism of metabolic signalling and protection (Dolder *et al.* 2003), the fate of mitochondria in heart and oxidative skeletal muscle cells would be left under the monopolized control of calcium. Under these metabolic circumstances an Olympic athlete would probably be dead before finishing the competition, because of mitochondrial

calcium overload and PTP opening, accelerated by ROS production by mitochondria (Brookes *et al.* 2004). Indeed, any attempt to increase the respiration rate more than 2-fold by calcium would necessarily imply a heavy overload of mitochondria by this potentially dangerous cation. Very fortunately, the regulation of mitochondrial function by the efficient protective mechanism of metabolic feedback channelling was chosen by nature long ago: the mitochondrial CK octamer appeared in very early stages of biological evolution (Ellington, 2001).

The importance of CK function is corroborated by the phenotype of transgenic CK knock-out mice. Mice that do not express either muscle-type cytosolic or sarcomeric MtCK, or both, exhibit, despite some remarkable compensations, like-enhanced muscle glycogen content and a massive increase in mitochondrial volume fraction (Steehgs *et al.* 1998), marked difficulties with peak force development and burst activity (van Deursen *et al.* 1993), as well as disturbances both in frequency and power output of voluntary movements (Momken *et al.* 2005). Studies with CK double knock-out mice have shown that the CK system is critically necessary to maintain optimal heart

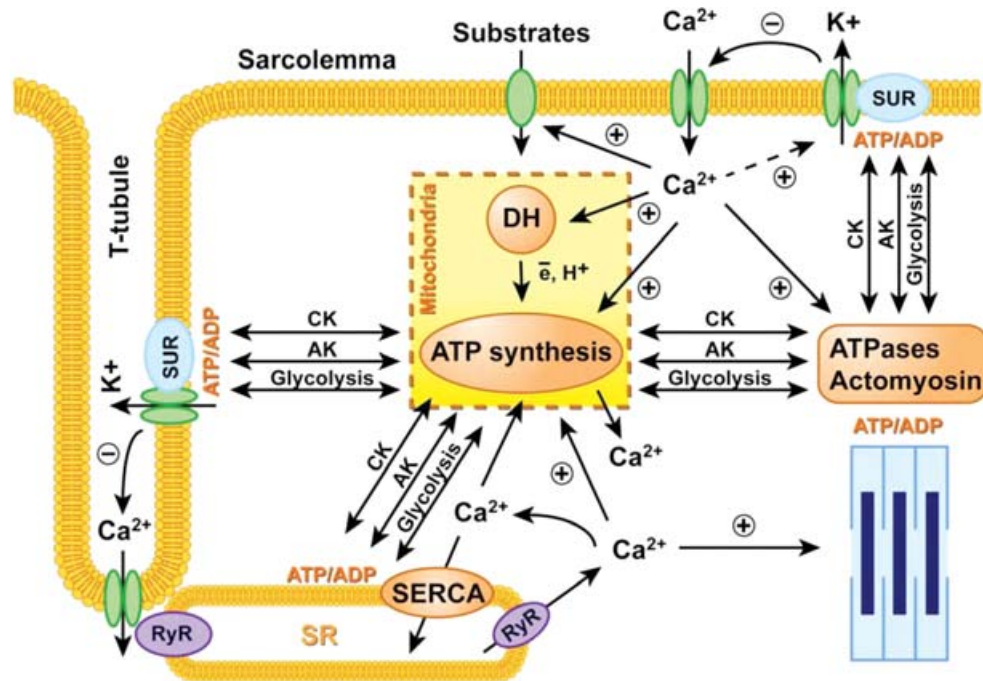


Figure 6. Cardiac excitation-contraction-energy coupling: synchronization electrical and metabolic pacing

Electrical pacing induced action potential and membrane depolarization causes Ca²⁺ influx through L-type Ca²⁺ channels in sarcolemma and T-tubules triggering Ca²⁺ release from intracellular stores in sarcoplasmic reticulum (SR) through ryanodine receptor-channels (RyR) and cardiomyocyte contraction. Interplay between CK, AK and glycolytic phosphotransfer relays, energetic modules (mitochondria and ATPases), metabolic sensors (K_{ATP} channel) and Ca²⁺ transients, generate metabolic pacing signals in synchrony with the electrical and functional activity to ensure cellular energetic homeostasis. After contraction intracellular Ca²⁺ is sequestered by SR Ca²⁺-ATPase (SERCA), by mitochondria, and removed from the cell by the Na⁺-Ca²⁺ exchanger and a Ca²⁺ pump in sarcolemma (see details in the text).

function at increased workloads (Spindler *et al.* 2004). The strongest phenotype of CK double knock-out mice is characterized by significant perturbations in intracellular calcium handling and muscle relaxation, emphasizing the physiological importance of the CK system for the energetics of intracellular calcium homeostasis in general and for the delivery of ATP to the energetically demanding Ca^{2+} -ATPase, in particular (Steeghs *et al.* 1998). Hearts with deficient cytosolic and mitochondrial CK isoforms exhibit impaired calcium homeostasis and increased susceptibility to ischaemia-reperfusion injury (Spindler *et al.* 2004). The M-CK gene knockout also shifts cellular energetics and signal delivery to K_{ATP} channels towards the glycolytic system, thus generating a phenotype with increased electrical vulnerability to disturbances in glucose metabolism (Abraham *et al.* 2002; Dzeja *et al.* 2004). Interestingly, fast-twitch CK-knockout skeletal muscles fatigue more slowly during repeated tetanic stimulation, probably due to an increase in mitochondrial volume and a decrease of crossbridge cycling as the result of actomyosin-ADP complex formation, which cooperatively promotes strong-binding crossbridges (Dahlstedt *et al.* 2000; Fukuda *et al.* 2000).

Adenylate kinase, whose phosphotransfer flux along with AMP concentration markedly increases under stress, contributes to the response of K_{ATP} channels under metabolic challenge (Carrasco *et al.* 2001). Deletion of the adenylate kinase *AK1* gene compromises nucleotide exchange at the metabolic sensing K_{ATP} channel site and impedes communication between mitochondria-generated signals and K_{ATP} channels, rendering cellular metabolic sensing defective (Carrasco *et al.* 2001). Hearts deficient in the cytosolic adenylate kinase (*AK1*) isoform have aberrant AMP and adenosine metabolic signalling, compromised ability to maintain nucleotide pools and increased vulnerability to injury (Pucar *et al.* 2002), suggesting that normal adenylate kinase function contributes to stress resistance in the heart. Double genetic disruption of the cytosolic CK (M-CK)- and *AK1*-catalysed phosphotransfer pathways compromises intracellular metabolic communication and energetic efficiency reducing the cellular capability to maintain total ATP turnover under functional load (Janssen *et al.* 2003b). Thus, new methodologies and transgenic gene manipulations provide the opportunity to decipher regulatory mechanisms that underlie cardiac bioenergetic homeostasis, and have revealed their significance for normal muscle function.

Due to the central importance of this efficient and protective metabolic feedback signalling system, its alteration in pathological states of the cell contributes significantly to the development of cardiac failure, either due to a decrease of total creatine content (Nascimben *et al.* 1996; ten Hove *et al.* 2005a), alteration of CK

expression (Nahrendorf *et al.* 2005), or changes of the coupling of CK in mitochondria and with MgATPases (Zoll *et al.* 2005; for review see Ventura-Clapier *et al.* 2004; Schlattner *et al.* 2005). Ingwall and colleagues have shown that the product of CK activity and total creatine content, $\text{CK} \times [\text{Cr}]$, decreases by a factor of 3–6 in cardiomyopathy and in failing human hearts (Nascimben *et al.* 1996). This is consistent with earlier data showing that replacement of creatine by its much less productive analogue guanidino propionic acid, given in diet, decreases by more than half the maximal work performance capacity and oxygen consumption of isolated working perfused heart (Kapelko *et al.* 1988). Also, significant wash-out of creatine during long periods of perfusion of frog hearts has been shown to lead to a hypodynamic state with a 2- to 3-fold decrease of contractile force, which can be reversed by uptake of creatine (Saks *et al.* 1976; Ventura-Clapier & Vassort, 1980). Very recently, it was shown in Neubauer's laboratory that hearts with undetectable levels of creatine and phosphocreatine due to knock-out of a key enzyme of creatine biosynthesis, lost the ability to respond to inotropic stimulation even at low workload, and showed markedly impaired recovery of heart function during ischaemia/reperfusion (ten Hove *et al.* 2005b). Momken *et al.* (2005) found recently that the double M-CK and Mt-CK knock-out mice showed only 10% of work capacity in voluntary exercise in running wheels, as compared with that of normal wild-type mice. These experimental results fit surprisingly closely with those of mathematical modelling (Fig. 4A) according to which, in the absence of creatine, the workload (the MgATPases, due to their inhibition by accumulating MgADP) and the rate of oxygen consumption are decreased by a factor of 4–5 (Vendelin *et al.* 2000). This model, however, does not yet include the AK and glycolytic systems and does not account for the adaptation phenomena in the CK knockout mice, which may increase the work capacity of the muscles.

Thus, effective cardiac work and fine metabolic regulation of respiration and energy fluxes need the organized and interconnected energy transfer and metabolic signalling systems. Direct transfer of ATP and ADP between mitochondria and different cellular compartments is not able to fulfil this important task efficiently.

Concluding remarks and future directions

The paradox of two apparently opposite conclusions concerning the role of calcium in regulating mitochondrial respiration that were reached in two different areas of bioenergetic research, i.e. the mitochondrial calcium cycle and the Frank-Starling mechanism, can thus be

resolved by structural and quantitative analysis of cellular energy transfer networks as described in this review. The data reviewed indicate the critical importance of two interrelated systems regulating mitochondrial respiration and energy fluxes in the cells (Fig. 6): (1) the structurally organized enzymatic modules and networks of the CK–AK–glycolytic systems communicating flux changes in non-equilibrium steady state, and (2) the secondary system based on cellular and mitochondrial calcium cycles, which adjusts the capacity of substrate oxidation and energy-transducing processes to meet increasing cellular energy demands. Moreover, the coupled creatine kinase, adenylate kinase and glycolytic reactions by communicating signals to the K_{ATP} channel, a sarcolemmal metabolic sensor, provide fine-tuned regulation of excitation–contraction and thus the calcium cycle within a cell. Such integration of energetic and ion signalling systems provides the basis for ‘metabolic pacing’, synchronizing electrical and mechanical activities with energy supply processes, which is fundamental for optimal function of the heart. The integrated scheme described in Fig. 6 provides a good example of the problems of molecular system biology applied to the heart – a new and rapidly developing area of scientific research.

In cardiac cells, the first system explains quantitatively the metabolic aspect of the classical Frank–Starling law of regulation of cardiac function and respiration under conditions of metabolic stability and unchanged calcium transients. In agreement with this conclusion, recent studies have shown that also in skeletal muscle the metabolic signals arising from the contractile sites play a more substantial role than cytoplasmic calcium in the signalling pathway to oxidative phosphorylation (Kindig *et al.* 2005b). The second system explains adrenergic modulation of cardiac cell function and energetics under stress (Berridge *et al.* 2003). By regulating the sarcolemmal metabolic sensor – K_{ATP} channels, the CK–AK–glycolytic network affects the excitation–contraction process and thus the calcium cycle of the cell (Fig. 6). Both systems may be activated simultaneously, as it is observed in the case of positive inotropy induced by β -adrenergic agents, when Frank–Starling curves are shifted upward (Starling & Visscher, 1926; Opie, 1998). These two more-or-less independent systems regulating cellular energetics have already been described separately by mathematical models (Vendelin *et al.* 2000; Saucerman *et al.* 2003); their integration into quantitative models of whole cell function and metabolism is an interesting and challenging perspective of molecular system biology. Structural, functional, genomic and computational analysis of enzymatic clusters and networks is yet another future challenge in cellular energetics. One of the most intriguing questions among them is that of the nature of local restrictions of the diffusion of adenine nucleotides in a highly structured cytosol in spite of the relatively

high rates of their diffusion in the intracellular bulk water phase (de Graaf *et al.* 2000). These diffusion restrictions result in compartmentation of adenine nucleotides and kinetic and thermodynamic inefficiency of energy-dependent processes, which may explain the necessity of energy transfer and metabolic signalling networks. Future developments to verify predictions made by theoretical considerations and by increasingly complex mathematical models should include new experimental methods allowing detection of small changes in the transduced metabolic signals (Cr, ADP, AMP, P_i) in critical cellular compartments, and new means of independent manipulation of energetic signals. This would provide a broader and more molecular understanding of regulation of cellular energetics and metabolic signalling, a rapidly growing area in cellular system biology.

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