

# Chapter 11

## Systems Level Regulation of Cardiac Energy Fluxes Via Metabolic Cycles: Role of Creatine, Phosphotransfer Pathways, and AMPK Signaling

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**Abstract** Integrated mechanisms of regulation of energy metabolism at cellular, tissue, and organ levels are analyzed from a *systems biology* perspective. These integrated mechanisms comprise the coordinated function of three cycles of mass and energy transfer and conversion: (1) the Randle cycle of substrate supply, (2) the Krebs cycle coupled with energy transformation in mitochondrial oxidative phosphorylation, and (3) the kinase cycles of intracellular energy transfer and signal transduction for regulation of energy fluxes. These cycles are extended and partially governed by information transfer systems like those linked to protein kinase signaling. In the heart, these cycles are closely related to the  $\text{Ca}^{2+}$  cycle during excitation–contraction coupling. According to the view of integrated metabolic cycles, the phosphocreatine/creatine kinase system represents a most important subsystem determining the efficiency of regulation of metabolic and energy fluxes in heart, brain, and oxidative skeletal muscles. It carries about 80 % of the energy flux between mitochondria and cytoplasm in heart. The substrate uptake, respiration rate, and energy fluxes are regulated in response to workload via phosphotransfer pathways and  $\text{Ca}^{2+}$  cycling. We propose integrated network mechanisms to explain the linear relationship between myocardial oxygen consumption and heart work output under conditions of metabolic stability (metabolic aspect of Frank–Starling’s law of the heart). The efficiency of energy transfer, force of contraction, and metabolic regulation of respiration and energy fluxes depend upon the intracellular concentration of total creatine, which is decreased in heart failure. The role of creatine, creatine kinase, and adenylate kinase phosphotransfer and AMP-activated protein kinase (AMPK) signaling systems and their interrelationship with substrate supply and  $\text{Ca}^{2+}$  cycles are analyzed. Finally, an introduction to the AMPK signaling network is provided with a particular emphasis on the heart in health and disease.

## 11.1 Introduction

In this chapter, we describe from a systems biology perspective the integration and regulation of substrate and energy supply in living organisms and the role of the creatine/creatine kinase (Cr/CK) system. Systems biology focuses on the mechanisms of interactions between system components at molecular, cellular,

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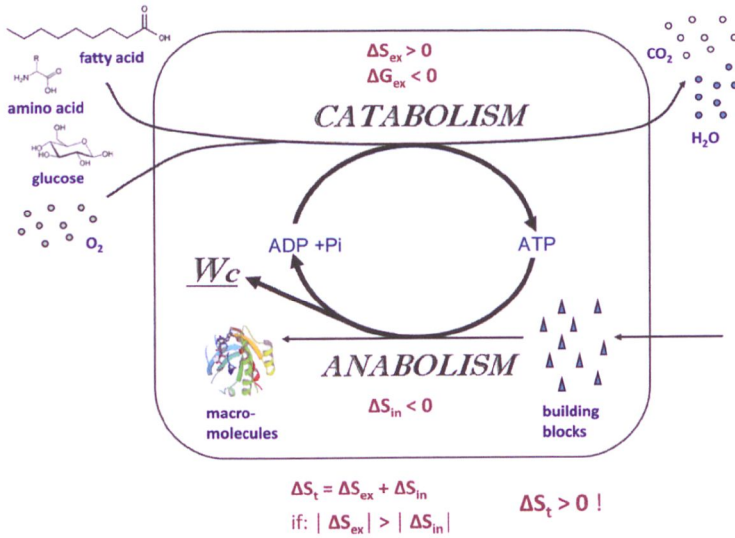
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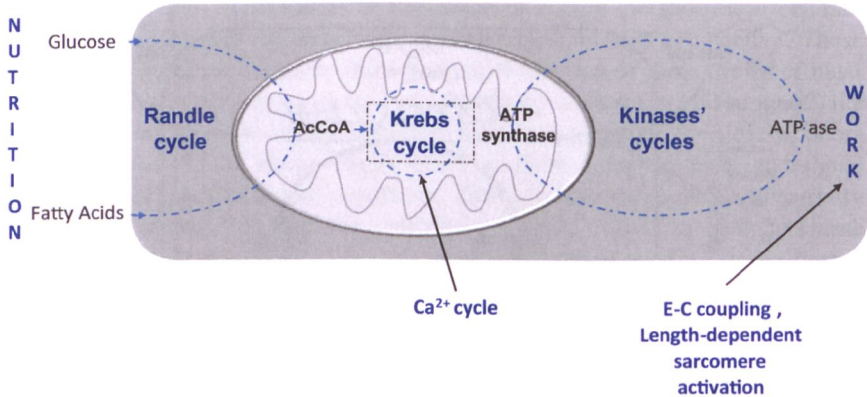
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**Fig. 11.1** General scheme of cellular metabolism. Catabolic reactions generating ATP (*top*), through coupling to anabolic reactions (biosynthesis, *bottom*) using ATP, maintain cell structural organization as an expression of the decrease of internal entropy ( $\Delta S_{in} < 0$ ) and are also the source of energy for cellular work ( $W_c$ ). Abbreviations:  $\Delta S_{ex}$  external entropy,  $\Delta S_{in}$  internal entropy,  $\Delta S_t$  total entropy,  $\Delta G_{ex}$  variation of Gibbs free energy. For further details, see text. Adapted from (Saks 2007) with permission

and organ levels, giving rise to biological function. As such, systems biology provides basic mechanistic insights about the principles that govern metabolic behavior in living systems. According to Schrödinger, the metabolic activity of living systems needs a continuous exchange of metabolites with the surroundings as a form of extracting free energy from the medium. This process enables cells and organisms to increase their internal organization such that they are able to perform biological work from anabolic reactions (Schrödinger 1944). An increase of internal order implies a decrease of entropy that should be compensated by an entropy increase in the environment. Catabolic and anabolic reactions are coupled to mediate biological work (e.g., muscle contraction) through processes of free energy conversion involving synthesis and utilization of ATP (Fig. 11.1). Coupling between cellular work, anabolism, and catabolism is achieved by cyclic processes involving mechanisms of feedback regulation. Herein, we introduce the theory of integrated metabolic cycles. Cycles of substrate supply (Randle cycle), intracellular energy conversion (Krebs cycle and mitochondrial oxidative phosphorylation), and phosphotransfer reactions (kinase cycles) constitute conspicuous examples of both substrate and energy provision and feedback regulation (Fig. 11.2). These cycles closely interact with calcium ( $Ca^{2+}$ ) cycling (Fig. 11.2). Among the kinase cycles, a key role is played by the Cr/CK system, adenylate kinase, and AMPK in skeletal muscle, heart, brain, and other cell types (Wallimann et al. 1992, 2011; Schlattner et al. 2006a, b; Schlattner and Wallimann 2004; Wallimann 1996, 2007; Saks



**Fig. 11.2** General representation of regulation of energy fluxes via metabolic cycles at the cellular level. The regulatory action that energy transfer cycles, such as the creatine kinase (CK) and adenylate kinase systems (AK), exert on fuel supply is realized through the Randle cycle and energy transforming Krebs cycle, coupled to oxidative phosphorylation. Any decrease in the use of intracellular energy diminishes Krebs cycle activity and tends to favor the accumulation of substrates

et al. 1978, 2007a, 2010, 2012; Saks 2007; Dzeja and Terzic 2003, 2009). In the heart, contraction is initiated by excitation-contraction coupling that includes processes linked to intracellular  $\text{Ca}^{2+}$  cycling (Bers 2002; Bers and Despa 2006). Under physiological conditions, contractile force and cardiac work are regulated by ventricular filling and sarcomere length-dependent mechanism (Frank-Starling's law) at constant amplitude of  $\text{Ca}^{2+}$  transients. A main regulatory motif of cardiac energy fluxes is represented by metabolic feedback regulation through local changes in Pi, ADP, AMP, Cr, and phosphocreatine (PCr) ratios (Saks et al. 2006a, 2010, 2012; Bose et al. 2003; Dos Santos et al. 2000; Aliev et al. 2012). Under conditions of adrenergic stimulation, cardiac  $\text{Ca}^{2+}$  cycling in the cytoplasm and mitochondria becomes most important for energy flux regulation (Balaban 2002; Griffiths and Rutter 2009; Tarasov et al. 2012; Glancy and Balaban 2012). Control and regulation of mitochondrial respiration by both adenine nucleotides and  $\text{Ca}^{2+}$  have been analyzed in an integrated model of cardiomyocyte function (Cortassa et al. 2009).

In this work, we aim to analyze regulatory interactions involved in the modulation of energy supply and demand in the network comprised by Randle and Krebs cycles and phosphotransfer pathways in the heart. Contribution of calcium cycling to the regulation of energy supply-demand in the heart has been extensively reviewed elsewhere (Balaban 2002, 2009a, b, 2012; Tarasov et al. 2012; Glancy and Balaban 2012). The synchronization of the mitochondrial network in cardiac cells is treated by Cortassa and Aon in Chap. 5.

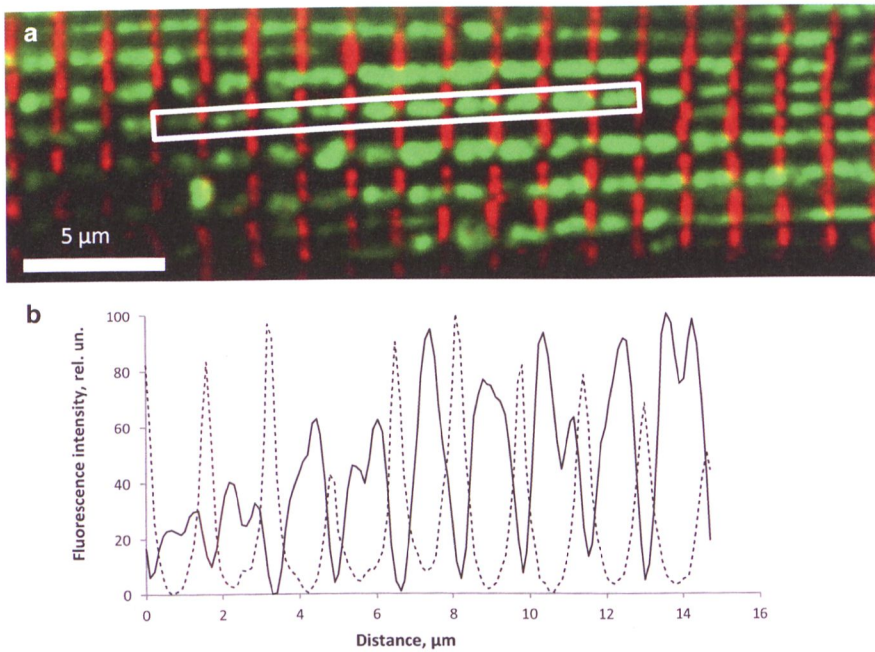


## 11.2 Structural Basis of Functional Organization of Cardiomyocyte Metabolism

In adult cardiac cells, mitochondria are localized at the A band level of sarcomeres between Z-lines close to T-tubular system and sarcoplasmic reticulum (SR). Estimation of the density distribution of mitochondria relative to their centers showed that neighboring mitochondria in cardiomyocytes are aligned according to a rectangle with distance between centers equal to  $1.97 \pm 0.43 \mu\text{m}$  and  $1.43 \pm 0.43 \mu\text{m}$  in the longitudinal and transverse direction, respectively (Vendelin et al. 2005). High temporal resolution analysis of mitochondrial dynamics in adult cardiomyocytes (one frame every 400 ms) revealed very rapid fluctuation of center positions that did not exceed the limit of the organelle (Beraud et al. 2009). These limited mitochondrial oscillations can be explained by inner membrane conformational changes likely elicited by changes in volume associated with energetic/redox states (Hackenbrock 1968; Mannella 2006). In vivo imaging of mitochondrial dynamics in cardiomyocytes showed separated individual organelles which do not fuse with each other (Gonzalez-Granillo et al. 2012). Figure 11.3 shows confocal images of mitochondria and  $\alpha$ -actinin distribution in cardiomyocytes from adult rats. In this figure the fluorescence immunolabelling of  $\alpha$ -actinin is used to mark sarcomeric Z-lines. Individual mitochondria regularly arranged between Z-lines can be visualized by flavoprotein autofluorescence (Fig. 11.3, green). The green fluorescence intensity profile shows the peaks distribution corresponding to mitochondrial fluorescence; the regions of “zero” intensity of  $\alpha$ -actinin (Fig. 11.3 red) indicate intermyofibrillar localization of mitochondria between Z-lines without apparent fusion/fission (Gonzalez-Granillo et al. 2012). Possibly, fusion can happen in perinuclear mitochondrial clusters (Kuznetsov and Margreiter 2009).

Regular arrangement and limited morphodynamics of mitochondria in adult cardiomyocytes are determined by the cytoskeletal architecture, which includes myofilaments, inter-myofilaments, microtubules, and other structural proteins. Tubulin is one of the constituent cytoskeletal proteins with structural, transport, and metabolic functions (see also Chap. 7). Herein, we will focus on the structural role of  $\beta$  isotypes of tubulin. Tubulin is a heterodimeric complex formed by two globular and two C-terminal tails (CTT) of  $\alpha$  and  $\beta$  proteins. Globular  $\alpha$  and  $\beta$  proteins can be polymerized into microtubules, while  $\alpha$  and  $\beta$  CTT can interact with other intracellular structures and proteins. Tubulin has additional binding sites that allow the filaments to join together laterally to form sheets of filaments. About 30 % of tubulins in adult cardiomyocytes are polymerized and 70 % are in the heterodimeric state (Tagawa et al. 1998). These two conformational states of protein are in a dynamic balance driven by polymerization–depolymerization processes (Sackett 2010). A study of the distribution of  $\beta$  tubulins by fluorescence confocal microscopy showed that  $\beta\text{IV}$  tubulin is polymerized creating a dense mesh of mainly longitudinally and obliquely oriented microtubules.  $\beta\text{III}$  tubulin co-localizes with  $\alpha$ -actinin in Z-lines while  $\beta\text{I}$  tubulin forms randomly dispersed short polymers





**Fig. 11.3** Fluorescence confocal microscopy of mitochondria and alpha-actinin distribution in adult rat cardiomyocyte. (a) Regular distribution of individual mitochondria as visualized by autofluorescence of flavoproteins (green color) in between Z-lines that are labeled with rhodamine immunofluorescent for  $\alpha$ -actinin (red color). (b) Analysis of fluorescence intensity along a selected line: dotted =  $\alpha$ -actinin; solid = flavoproteins. Note that peaks of green fluorescence intensity corresponding to mitochondria are seen in the regions of “zero” intensity of red  $\alpha$ -actinin fluorescence. Reproduced from (Gonzalez-Granillo et al. 2012) with permission

and dimers, and  $\beta$ II tubulin co-distributes with mitochondria (Saks et al. 2012; Gonzalez-Granillo et al. 2012; Guzun et al. 2011a, 2012). These findings are in agreement with data published first in 1990 by Saetersdal et al. regarding the link between  $\beta$  tubulin and mitochondria as revealed by immunogold labeling (Saetersdal et al. 1990). According to this study,  $\beta$  tubulin interacts with mitochondria through the outer membrane (MOM) creating links between the organelle and other cellular structures. The contribution of other cytoskeletal proteins to structural and functional interactions with mitochondria is under intensive investigation. Desmin and plectin are capable of interacting with voltage-dependent anion channel (VDAC) at MOM (Capetanaki et al. 2007; Capetanaki 2002; Liobikas et al. 2001; Schroder et al. 2002). The 1b isotype of plectin of cardiomyocytes co-localizes with mitochondria via direct interaction with VDAC, whereas plectin 1d isotype is specifically associated with sarcomeric Z-disks (Schroder et al. 2002).

Recently it has been proposed that the T-tubular system, which represents a network of tubular extensions from the sarcolemma, plays an important role in the

structural organization of cardiac cell metabolism. The T-tubular system of rat ventricular cells creates a regular arrangement at the level of Z-line and along myofibrils (Fig. 11.4c) (Soeller and Cannell 1999). This system becomes disorganized with time in cardiac cells in culture. The functional role of T-tubules was described to provide a rapid inward spread of electrical excitation and  $\text{Ca}^{2+}$  influx that triggers  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum, as well as supply of each mitochondrion with oxygen and substrates. By using electron tomography (Hayashi et al. 2009) identified anatomical couplings between opposing membranes of T-tubules and sarcoplasmic reticulum (SR), these forming so-called Calcium Release Units (CRU). A close localization of mitochondria and CRU favors  $\text{Ca}^{2+}$  and metabolite microcompartmentation (Saks et al. 2012). Individual mitochondria localize at the level of the A-band of sarcomeres and at the Z-line they are in close contacts with jSR and the T-tubular system forming CRUs (Fig. 11.4b). This junctional cisterns of arrangement separates mitochondria from each other, also making their fusion unlikely. The 3D reconstruction of the T-tubular system in cardiac cells (Soeller and Cannell 1999) appears as an elaborated and effective system of  $\text{Ca}^{2+}$ , substrate, and oxygen supply from the extracellular medium. Its discovery about a decade ago profoundly changed our knowledge of the heart cell structure and the implications for metabolic regulation. As a matter of fact, according to this architecture no distinction is possible between intermyofibrillar and subsarcolemmal mitochondria, since both are in close contact with the T-tubular system. This is in agreement with results obtained from kinetic studies (Saks et al. 2012) and the fact that no electrical conduction occurs between individual mitochondria in cardiomyocytes (Beraud et al. 2009; Kuznetsov et al. 2009; Collins and Bootman 2003; Nivala et al. 2011; Zorov et al. 2000). Simultaneous measurements of sarcomere and mitochondrial dimensions in situ along the longitudinal axis of cardiomyocytes identified mitochondria as micron-sized spheres localized between sarcomeres and distributed throughout the cell in a crystal-like lattice without any visible fusion. In this organized lattice, transient mitochondrial depolarizations (flickers), elicited by ROS-induced opening of anion channels in the inner membrane, may propagate in cells as depolarization waves (Nivala et al. 2011; Yaniv et al. 2011). However, electron tomographic studies clearly revealed that there is no mitochondrial reticulum in cardiac cells; instead a regular lattice containing 5,000–10,000 single mitochondria seems to prevail (Nivala et al. 2011). In the heart, this forms the structural basis of the mitochondrial network described by Cortassa and Aon in Chap. 5. Taken together, all the data described above indicate that mitochondrial respiration depends upon localized events in their vicinity. These structurally organized functional domains—dubbed Intracellular Energetic Units (ICEUs) (Saks 2007; Saks et al. 2001, 2012) (Fig. 11.5)—comprise sites of ATP hydrolysis (myofibrillar ATPases, sarcoplasmic reticulum ATPase (SERCA), ion pumps) connected to ATP synthesis through phosphotransfer networks. Energy transduction within ICEUs involving the Randle and Krebs cycles of fuel supply and oxidative phosphorylation are governed by energy-demanding reactions. Next, we analyze cardiac energy metabolism from the perspective of regulatory interactions occurring in metabolic cycles.



## 11.3 Substrate Supply and Its Regulation (Randle and Krebs Cycles)

### 11.3.1 *Mechanisms of Regulation of Fatty Acids Oxidation in Heart Muscle*

Fatty acids are released from triacylglycerol (TAG) by activated lipoprotein lipase (LPL) and transferred in the cytoplasm bound to proteins. Free fatty acid transfer across mitochondrial membranes consumes ATP involving FFA conversion into an Acyl-CoA derivative and the transport-competent acyl-carnitine form by carnitine palmitoyl transferase (CPT). The MOM-localized CPT1 targeted by malonyl CoA inhibition constitutes an important regulatory step of  $\beta$ -oxidation of FAs ( $\beta$ -FAO) (Fig. 11.4) (Saks et al. 2006b).  $\beta$ -FAO is linked to the citric acid cycle and oxidative phosphorylation through  $\text{NAD}^+$ , FAD, and acyl-CoA. The NADH generated by the Krebs cycle and  $\beta$ -FAO is oxidized in the electron transport chain. Increased ATP utilization elicits ATP synthesis driven by the proton motive force, thus decreasing the  $\text{NADH}/\text{NAD}^+$  ratio. Oxidation of the NADH pool increases the flux through the Krebs cycle through  $\text{NAD}^+$ -dependent isocitrate and  $\alpha$ -ketoglutarate dehydrogenases, thus decreasing acetyl-CoA (AcCoA) levels.  $\text{NAD}^+$  can also be reduced in  $\beta$ -FAO catalyzed by  $\beta$ -hydroxyacyl-CoA dehydrogenase and in the glycolytic pathway catalyzed by glyceraldehyde phosphate dehydrogenase (GAPDH). However, the transfer of NADH reduction potential from glycolysis towards the mitochondrial matrix via the malate–aspartate shuttle, being slower than direct  $\text{NAD}^+$  use by  $\beta$ -FAO, will prioritize the latter one (Kobayashi and Neely 1979). Thus, the GAPDH dependence on cytoplasmic  $\text{NADH}/\text{NAD}^+$  ratio associated with the slow kinetics of malate–aspartate shuttle will rather slow down glycolysis. An increase in the rate of AcCoA utilization by the Krebs cycle will thus increase  $\beta$ -FAO. An accumulation of AcCoA does not influence significantly the rate of  $\beta$ -FAO due to the equilibrium constant of the reversible thiolase reaction which is in favor of AcCoA production (Neely and Morgan 1974).

At low ATP demand (decreased workload), the high  $\text{NADH}/\text{NAD}^+$  ratio slows down the flux through  $\text{NAD}^+$ -dependent dehydrogenases, thus decreasing the rate of AcCoA oxidation through the Krebs cycle. An increased intra-mitochondrial AcCoA level is thought to favor its transfer towards the cytoplasm where it is converted into malonyl-CoA, an inhibitor of CPT-1-controlled FA transport into mitochondria. Malonyl-CoA levels are also controlled by acetyl-coA carboxylase (ACC), a cytosolic enzyme catalyzing conversion of AcCoA into malonyl-CoA, whose inactivation by AMPK during energy stress relieves CPT1 inhibition.

Preferential utilization of FAs involves inhibition of glucose transport, phosphofructokinase (PFK), and pyruvate dehydrogenase (PDH) reactions (Hue and Taegtmeyer 2009; Taegtmeyer 2010; Taegtmeyer et al. 2005). Glucose transport in muscle cells is realized through GLUT4, the expression of which in the sarcolemma is regulated by insulin and other signals. Increased  $\text{NADH}/\text{NAD}^+$  and





AcCoA /CoA ratios inhibit PDH. Their inhibitory effect is realized through pyruvate dehydrogenase kinase (PDK) that phosphorylates and inhibits PDH (Randle 1998). Citrate that escapes oxidation in the Krebs cycle is transported to the cytosol where it inhibits PFK and glycolysis (Hue and Taegtmeyer 2009; Taegtmeyer 2010; Taegtmeyer et al. 2005).

Cell signaling via AMPK provides a parallel control of most of these processes, including substrate uptake via fatty acid and glucose transporters and flux via  $\beta$ -FAO and glycolysis (see Sect. 5.5). Activation of AMPK during energy stress situations stimulates all these activities.

Physiologically, the significance of the Randle cycle is to ensure the provision of FAs to high-energy demanding organs such as muscle and liver. Also, glucose is directed to organs such as brain, red blood cells, and other tissues dependent upon glucose oxidation and possessing relatively small stores of glycogen.

### 11.3.2 Which Substrate Is Better: Reductionism Versus Systems Biology

Living cells extract and transform energy from different sources distributing them between organs, as a function of their energy needs and metabolic potential. Unfortunately, there is not yet consensus on evaluating the amount of energy that may be extracted from different carbon sources. A reason for this is differences between reductionistic and systems biology type of approaches. The reductionist explanation of the competitive use of different energy sources by distinct organs is based on the oxygen needed to oxidize the different substrates and considerations of coupling of oxidative phosphorylation. All electrons from NADH produced in aerobic catabolism (i.e., from glycolysis and fatty acid oxidation) enter the respiratory chain via complex I, or electrons from FADH<sub>2</sub> formed in  $\beta$ -FAO are carried via electron transferring flavoprotein and complex III (Fig. 11.4), resulting in lower ATP/O ratio. In this way, the yield of 38 ATP for 12 atoms of oxygen consumed ( $P/O = 3.16$ ) for glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) oxidation and the yield of 129 ATP for 46 atoms of oxygen consumed ( $P/O = 2.8$ ) for palmitic acid (C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>) oxidation are assumed to be sufficient to conclude that glucose is the preferential fuel for living organisms. This conclusion is further corroborated by measurements of oxygen consumption by direct calorimetry. When one liter of oxygen is used to burn substrates, the amount of energy obtained is 5.19 kcal/LO<sub>2</sub> for glucose and 4.81 kcal/LO<sub>2</sub> for palmitic acid (Leverve et al. 2006). However, these calculations

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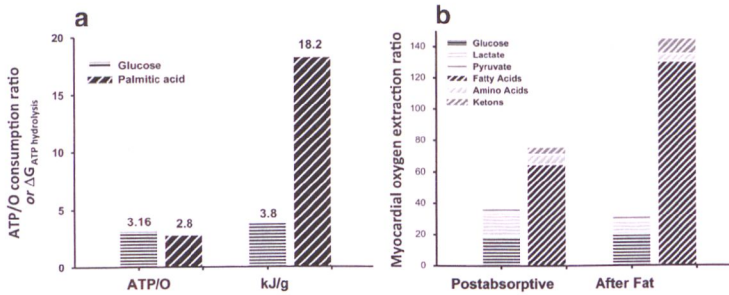
**Fig. 11.4** (continued) would inhibit hexokinase (HK), and decrease glucose transport. *G6P* glucose 6-phosphate, *HK* hexokinase, *PFK* phosphofructokinase, *GLY* glycogen, *F1,6diP* fructose-1,6-bisphosphate, *GAPDH* glyceraldehyde 3 phosphate dehydrogenase, *1,3DPG* 1,3 diphosphoglycerate. AMPK signaling (orange) controls among others substrate uptake and flux via glycolysis and fatty acid oxidation under conditions of starvation, hypoxia and other triggers of energy stress. For details see text. Modified from (Saks et al. 2012) with permission



do not take into account that under aerobic physiological conditions oxygen is not a limiting factor for energy metabolism, but instead that there are many other factors to be taken into account in the whole system. And these factors were indeed taken into account by nature. Regarding the fuel supply to such a high-energy demanding organ as is the heart, Clark and collaborators were the first to show that glucose constituted less than 1/4 of the substrates oxidized by the isolated working frog heart (Clark et al. 1937). These authors were not able to figure out which substrate(s) were responsible for consuming the remnant oxygen. In 1954, Bing and collaborators showed that the respiratory quotient (RQ,  $VCO_2/VO_2$ ) in post-absorptive state was about 0.7–0.75 while studying oxygen utilization during the aerobic metabolism of fats, ketones, and amino acids by human heart (Bing et al. 1954). This ratio was unchanged following overnight fasting but increased above 1 after ingestion of a high fat diet. The authors assumed that this increase could be due to utilization of intramuscular triacylglycerol (TAG) stores (Bing et al. 1954). Similar data were obtained in skeletal muscle. The average respiratory quotient ( $VCO_2/VO_2$ ) of muscular tissue taken from de-pancreatized dogs was about 0.7 (Bing et al. 1954).

In the case of working heart, the preferential energy supply by FA can be understood from calculations specifying energy needs to realize work, energy content of different substrates per unit mass, and kinetics of reactions in Randle and Krebs cycles, rather than by oxygen consumed for oxidizing different fuels. A heart contracting with a frequency of 70 bpm exhibits a stroke volume of 0.07 L (i.e., cardiac output—5 L/min) that supports a pressure of 13 kPa (equivalent of 120/70 mm Hg) and realizes a work equal to 65 J/min or 93.6 kJ/day. ATP hydrolysis in the actomyosin reaction releases about 60 kJ/mol under physiological conditions. For the heart to accomplish a work equivalent to 100 kJ/day about 2.8 mol of ATP are needed ( $n = W/\Delta G_{ATP}$  corrected for the reaction efficiency that in the case of actomyosin is about 60 %). This amount of ATP can be obtained from the oxidation of 0.074 mol glucose or 0.02 mol of palmitic acid. For glucose, supplemented with an equivalent molecular weight of 10 mol of water, 26.5 g glucose should be oxidized by the heart to perform work equivalent to 100 kJ/day. For palmitic acid only 5.5 g of this FA are necessary to perform a similar amount of work. Thus, the content of free energy per gram of mass that can be released during oxidation and converted into chemical energy in the form of ATP is much higher for FAs than for carbohydrates due to the much higher content of non-oxidized  $-C-C-$  and  $-C-H$  chemical bonds. Depending on the amount of bound water the difference in carbohydrates can range from three- to ninefold (Newsholme and Start 1973) (Fig. 11.5b). Thus, the kinetics of mass transfer in substrate supply is much more favorable when FAs, as compared to glucose, are used as substrates. And this fact explains the choice made by nature: heart and oxidative skeletal muscle clearly prefer FAs as substrates (Fig. 11.5a). Their preferred utilization by heart and oxidative muscle is achieved by multiple regulatory mechanisms involved in the Randle and Krebs cycles (Fig. 11.4).

Randle et al. (1963) were the first to propose the concept of selective supply of FAs over glucose for heart muscle (Randle et al. 1963). The glucose–FA cycle or



**Fig. 11.5** The role of fatty acid oxidation in metabolism. (a) (i) ATP synthesis to oxygen consumption ratio in mitochondria for glucose and palmitate oxidation and (ii) the Gibbs free energy of ATP hydrolysis from the actin–myosin reaction obtained from the oxidation of one gram of glucose in comparison with the oxidation of one gram of palmitate. (b) Comparison of the myocardial oxygen extraction ratio of carbohydrates (glucose, pyruvate, and lactate) and non-carbohydrates (fatty acids, amino acids, ketones) in a post-absorptive state and after ingestion of FAs. In both states FAs oxidation is the prevalent source of energy for the heart [adapted from Bing et al. (1954) with permission]

Randle cycle outlined the restrictions imposed on muscle glucose metabolism by FA oxidation (Randle et al. 1963). Further mechanisms of regulation of the glucose–FA cycle in working heart were described by Neely and Morgan (1974) with new insights being revealed since then (Hue and Taegtmeyer 2009; Taegtmeyer 2010; Taegtmeyer et al. 2005). These mechanisms account for changes in the kinetics of fuels supply, mass transfer, and transformation including glucose transport and glycolysis, FA transport,  $\beta$ -FAO, and the Krebs cycle in response to variations in respiration rates and NADH oxidation.

## 11.4 Phosphotransfer Pathways (Kinase Cycles)

### 11.4.1 Creatine Biosynthesis and Transmembrane Transport

Creatine biosynthesis occurs in a two-step reaction; first, in the kidney and in pancreas, the amino acids arginine and glycine are combined to form guanidino acetic acid (GAA) by the enzyme AGAT (arginine-glycine amino-transferase), and second, in the liver, where GAA, taken up from blood serum via GABA-2 (gamma-aminobutyric acid transport) (Tachikawa et al. 2012), is methylated to generate Cr by GAMT (guanidine-acetic acid methyltransferase) using SAM (S-adenosine-methionine) as a substrate (Wyss and Kaddurah-Daouk 2000). Creatine synthesized in the liver is released into the bloodstream by a still unknown mechanism. Since creatine is not produced in significant amounts in, e.g., heart, brain, skeletal, and smooth muscle, where it plays an important functional role, it has to be imported by these tissues from blood serum, using a specific creatine transporter (CRT) (Beard

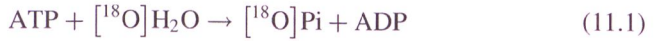


and Braissant 2010). In this way, creatine participates in the regulation of metabolism at the organ level. An increase in total Cr and PCr in cells also increases the PCr/ATP ratio and thus energy charge (Wallimann et al. 2011). Mutations in either of the genes coding for AGAT, GAMT (endogenous creatine synthesis), or CRT (creatine transport) in humans lead to the so-called creatine deficiency syndrome with a severe neuromuscular and neurological phenotype including developmental delay of expressive language and cognitive speech, mental retardation, autistic-like behavior, epilepsy, and brain atrophy (for review, see (Stockler et al. 2007)).

#### ***11.4.2 Direct Measurement of Energy Fluxes: Principal Role of the Phosphocreatine Pathway in Energy Transfer in the Heart***

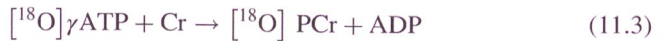
While Cr has been known for 175 years after its discovery by Michel Chevreul, the hypothesis of the PCr pathway was formulated by Samuel Bessman (Bessman and Carpenter 1985; Bessman and Fonyo 1966; Bessman and Geiger 1981) and independently by Martin Klingenberg (1970, 1976, 2008; Wallimann 1975; Turner et al. 1973; Saks et al. 1978) about 50 years ago. An important factual basis of this hypothesis is given by the observation made by Belitzer and Tsybakova (1939), who showed that Cr addition stimulated respiration in skeletal muscle homogenates, resulting in PCr production (Belitzer and Tsybakova 1939). A fundamental contribution to the existence of a PCr pathway of energy transfer in heart, muscle, brain, and other tissues was been made by Theo Wallimann's group. They showed that different CK isoenzymes belong to different compartments, with MtCK in mitochondria and cytosol and MM-CK in myofibrils and the membrane of sarcoplasmic reticulum. They also resolved the atomic structure of CKs and characterized interaction mechanisms with neighboring structures (Wallimann et al. 1992, 2007; Schlattner et al. 1998, 2006a, b; Schlattner and Wallimann 2004; Eder et al. 1999, 2000; Fritz-Wolf et al. 1996). MM-CK was also shown to localize in the sarcolemmal membrane (Saks et al. 1977). Such in vivo compartmentation of CK and ATP in muscle cells represents the cellular basis of the CK cycle, one of the phosphotransfer pathways of energy transport (Wallimann et al. 1992, 2007; Schlattner et al. 2006a, b; Schlattner and Wallimann 2004; Saks 2007, 2008, 2009; Aliev et al. 2012; Saks et al. 2007b). Detailed functional studies combining the use of mathematical modeling with experimental data have shown that within myofibrils, and in the subsarcolemmal area, the diffusion coefficient for ATP is decreased by factor of  $10^5$  as compared to water solution (Abraham et al. 2002; Alekseev et al. 2012; Selivanov et al. 2004). Diffusion limitations result in ATP compartmentation in cells, where the local ATP and ADP pools are connected by the phosphotransfer pathways. An equally important and fundamental contribution was been made by Dzeja and Terzic groups who measured quantitatively, using an isotope tracer method, energy fluxes between different cellular

compartments involving kinase cycles (Dzeja and Terzic 2003, 2009; Dzeja et al. 1999; Nemutlu et al. 2012). Most effective and informative in bioenergetic studies of phosphoryl transfer has been the use of  $^{18}\text{O}$  transfer (see the Chap. 6). This method is based on the following two reactions: ATP hydrolysis by water molecules containing  $^{18}\text{O}$  and ATP resynthesis with formation of  $[^{18}\text{O}]\gamma\text{ATP}$  (Dzeja and Terzic 2009; Nemutlu et al. 2012):



Paul Boyer used this method for studying the ATP synthase reaction (Boyer 1997). Inclusion of  $[^{18}\text{O}]\text{Pi}$  into  $[^{18}\text{O}]\gamma\text{ATP}$  in the presence of uncouplers led him to the conclusion of the rotational binding change mechanism of mitochondrial ATP synthesis. Nelson Goldberg, Petras Dzeja, André Terzic, and coworkers have successfully applied this method for studying the kinetics of phosphoryl-transfer reactions and energy fluxes *in vivo* by measuring the rates of the following reactions (Dzeja and Terzic 2003, 2009; Nemutlu et al. 2012):

Creatine kinase phosphotransfer:



Adenylate kinase phosphotransfer:

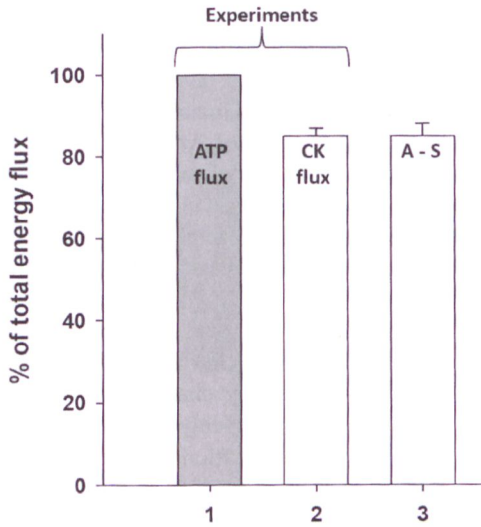


Glycolytic phosphotransfer:



If a direct transfer of ATP from mitochondria to MgATPases happens together with its immediate hydrolysis for contraction as sometimes proposed in the literature, only isotope transfer reactions 1 and 2 could be observed. In an excellent series of studies Dzeja's group showed that in normal cardiac cells about 80–85 % of phosphoryl groups are transferred out from mitochondria by the PCr flux, and about 10–15 % by adenylate kinase, with a minor contribution by glycolysis (Dzeja et al. 1999). In the heart, these fluxes increase linearly with workload energy demand under conditions of the Frank–Starling law (Saks et al. 2007c). Figure 11.6 shows that PCr fluxes measured experimentally can be quantitatively simulated with a mathematical model of compartmentalized energy transfer (Dos Santos et al. 2000; Aliev et al. 2012; Aliev and Saks 1997; Vendelin et al. 2000). This model was based on the experimental data obtained in studies of mitochondrial PCr synthesis in permeabilized cardiomyocytes. The role of the adenylate kinase system becomes important in hypoxia and pathological situations (Dzeja et al. 1999).





**Fig. 11.6** Comparison of experimental data of energy flux measurements with results of simulations by mathematical models. ATP flux: the rate of ATP synthesis in mitochondria; CK flux: energy flux carried into cytoplasm by phosphocreatine measured experimentally by the  $^{18}\text{O}$  transfer method [data summarized from Dzeja and Terzic (2003), Dzeja et al. (1996, 2001, 2007, 2011a), Pucar et al. (2001)]; A-S: Aliev and Saks models of compartmentalized energy transfer (Dos Santos et al. 2000; Aliev and Saks 1997). The mathematical model of the compartmentalized energy transfer system in cardiac myocytes includes mitochondrial synthesis of ATP by ATP synthase, PCr production in the coupled MtCK reaction, the myofibrillar and cytoplasmic CK reactions, ATP utilization by actomyosin ATPase during the contraction cycle, and diffusional exchange of metabolites between different compartments. The model gives a good fitting with the experimental data, showing that about 85 % of energy produced in mitochondria as ATP flux is transferred out of mitochondria as PCr flux, in agreement with the abundant experimental data reported by Dzeja and colleagues

Recently this method has been used in quantitative studies of metabolic cycles in human health and disease (Dzeja et al. 2011a).

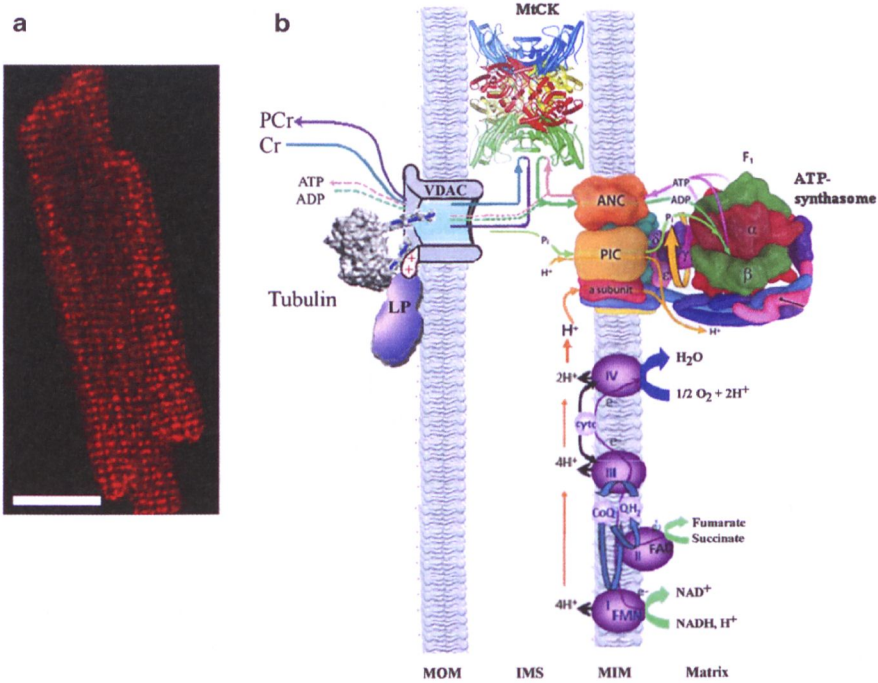
### ***11.4.3 Intracellular Energetic Units and Mitochondrial Interactosome: Local Signaling and Frank–Starling Law***

In addition to the fundamental structural data from Wallimann and Schlattner and energy flux determinations by Dzeja and Terzic, another important question concerns the cellular mechanisms involved in the function of CKs and other phosphotransfer pathways. This question was addressed by the group of Valdur Saks utilizing permeabilized cells that enable the study of mitochondrial function in their natural environment (Saks et al. 1991, 1998, 2007a, d; Saks and Strumia

1993). A central bioenergetic question in muscle cells relates to the mechanism of PCr synthesis in mitochondria. This question arises because the equilibrium and kinetic constants of all CK isoforms would favor only the resynthesis of MgATP from PCr and MgADP (Saks et al. 2010; Guzun et al. 2009). Kinetic information available is in agreement with the role of MM-CK at the sites of local ATP regeneration in myofibrils and membranes of sarcolemmal and sarcoplasmic reticulum, but this is not the case for PCr synthesis in mitochondria. More insight can be obtained from the classical problem of cardiac physiology—the metabolic aspect of the basic Frank–Starling law of the heart (Saks et al. 2006c, 2012). Discovered in 1914–1926, the Frank–Starling law states that under physiological conditions contractile force, cardiac work, and the rate of oxygen consumption increase manifold with the filling of the left ventricle (Starling and Visscher 1927). Later it was found that this occurs without any changes in the ATP and PCr levels (metabolic stability) and  $\text{Ca}^{2+}$  transients (Neely et al. 1972; Balaban et al. 1986). The latter observation excludes any explanation involving a mechanism of control of mitochondrial respiration by changes in intracellular  $\text{Ca}^{2+}$ . A  $\text{Ca}^{2+}$ -mediated mechanism may be important only in the case of adrenergic activation of the heart (Tarasov et al. 2012; Balaban 2012). Assuming that ATP, ADP, PCr, and Cr are related through equilibrium relationships, the observation of metabolic stability was interpreted to exclude any other explanation of workload dependence of cardiac oxygen consumption than a mechanism involving the control of mitochondrial respiration by ADP or Pi only. The popular assumption of CK equilibrium, as in a mixed bag of enzymes (Wiseman and Kushmerick 1995), however, is in contradiction with the experimental evidence (Saks 2008; Guzun and Saks 2010). This includes recent high-resolution  $^{31}\text{P}$  NMR experiments showing that the major part of adenine nucleotides, notably ATP in muscle cells, exists associated with macromolecules and that free ADP may be only transiently present in the cytoplasm (Nabuurs et al. 2010, 2013). We have shown that both high PCr fluxes in the heart detected by Dzeja and collaborators (Dzeja and Terzic 2003, 2009; Dzeja et al. 1999; Nemutlu et al. 2012) and the linear dependence of the rate of oxygen consumption on cardiac work may be explained by local signaling and metabolic channeling of adenine nucleotides in nonequilibrium CK reactions (Saks et al. 2012; Guzun et al. 2009; Timohhina et al. 2009). Actually, CK can catalyze within the same cell either the forward or the backward reaction depending on in which microcompartment the enzyme is located and where it functions as part of different multienzyme complexes.

Mechanisms involving the interaction of mitochondria and CKs with other cellular structures and multienzyme complexes are central for understanding metabolic stability in the heart. This implies a different perspective in the framework of systems biology. Figure 11.7a shows the localization of the tubulin isotype  $\beta\text{II}$  following the pattern of mitochondrial distribution in cardiac cells (Saks et al. 2012; Gonzalez-Granillo et al. 2012; Guzun et al. 2011b, 2012). Tubulin  $\beta\text{II}$  is part of the heterodimer tubulin that binds to VDAC in MOM, thus modulating the close probability of this channel specifically so that it is permeated by Cr or PCr but limited for ATP or ADP (Guzun et al. 2009; Timohhina et al. 2009). In cardiac





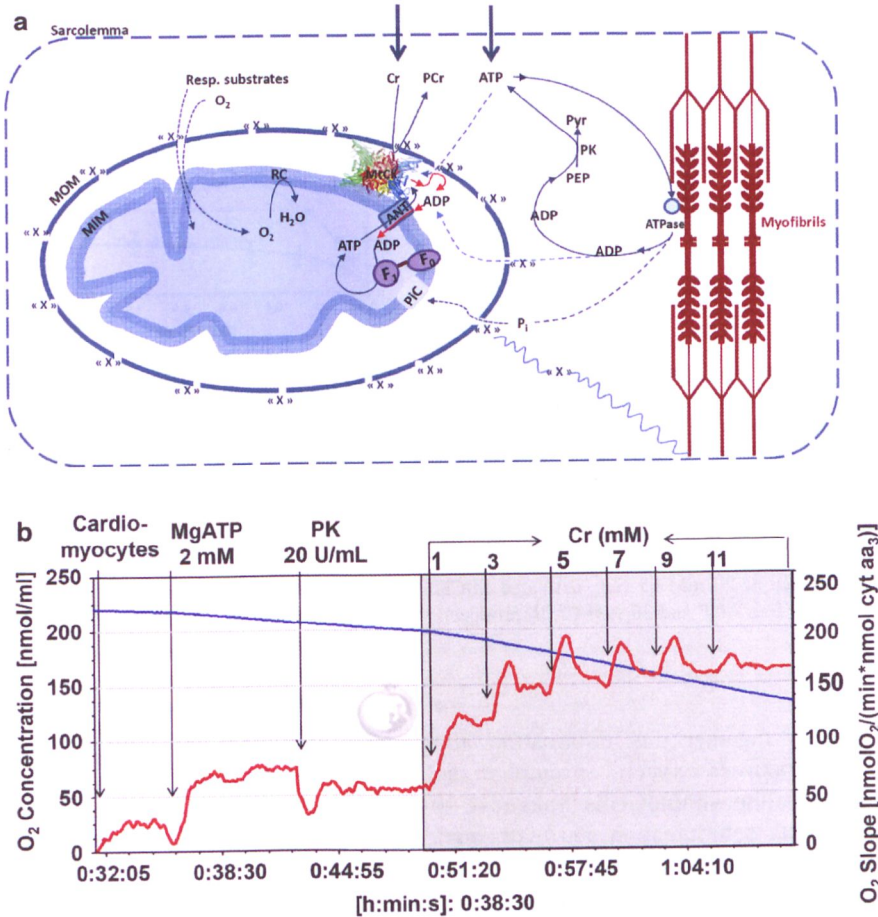
**Fig. 11.7** Mitochondrial Interactosome. (a) Confocal image of a cardiomyocyte labeled with MitoTracker Red for mitochondria; scale bar 14  $\mu\text{m}$ . (b) Scheme depicting the mitochondrial interactosome, a macromolecular complex formed by the ATP synthasome, in turn constituted by ATP synthase (subunits in different colors), adenine nucleotide translocase (ANC, orange), inorganic phosphate carrier (PIC, yellow), coupled to the respiratory complexes (I-IV, purple circles) in the mitochondrial inner membrane (MIM), octameric mitochondrial CK (MtCK, backbone structure with dimers in different color) in the intermembrane space (IMS) and the voltage-dependent anion channel (VDAC, gray-blue) in the mitochondrial outer membrane (MOM) interacting with cytoskeletal proteins tubulin (gray, surface structure representation) and putative linker protein (LP, purple). Metabolite fluxes are indicated by arrows in different colors. For ATP synthase, subunits of the  $F_1$  part (greek letters) and  $F_0$  part (latin letters) are indicated, as well as the rotation of the rotor (yellow arrow). For the respiratory chain, proton pumping ( $\text{H}^+$ , yellow arrows) and some redox centers (FMN, FAD) are indicated, as well as the two electron carriers coenzyme Q ( $\text{CoQ}/\text{CoQH}_2$ ) and cytochrome c (cytc). Adapted from (Timohhina et al. 2009) and (Schlattner et al. 2009) with permission. Art work of the ATP synthasome in this figure was reproduced with kind permission from P.L. Pedersen and is the result of the combined efforts of Drs. Young H. Ko and David J. Blum; MtCK structure and membrane topology is reproduced from (Schlattner et al. 2006b) with permission

cells, the heterodimeric tubulin  $\alpha\beta\text{II}$  and VDAC form a supercomplex with MtCK and the ATP synthasome—the mitochondrial interactosome (MI) (Fig. 11.7b) (Timohhina et al. 2009). Within this supramolecular structure, ATP and ADP cycle between ATP synthasome and MtCK maintaining oxidative phosphorylation effectively coupled to the synthesis of PCr. In the MI, MtCK functions

unidirectionally toward PCr synthesis utilizing mitochondrial ATP supplied by ANT (direct channeling). This process moves ADP back into mitochondria, because of the differential permeability of VDAC in interaction with tubulin that impedes ADP release from mitochondria. These coupled reactions of oxidative phosphorylation and PCr synthesis in MI are effectively regulated by Cr (Fig. 11.8). In the presence of an extra-mitochondrial ADP trapping system (pyruvate kinase, PK; phosphoenolpyruvate, PEP), Cr addition rapidly increases the respiration rate to its maximal value, revealing a preferential accessibility of the ADP produced by MtCK to matrix ATPase, not to the cytosolic trapping system. Metabolic control analysis of mitochondrial respiration in permeabilized cardiac cells showed high flux control coefficients (FCC) for reactions involving ADP recycling coupled to MtCK and PCr production (Fig. 11.9a). Actually, the sum of control coefficients exceeds the theoretical value for linear systems by a factor of 4 (Tepp et al. 2011). This can be interpreted in terms of MtCK-controlled reactions in MI acting as very effective amplifiers of metabolic signals from cytoplasm (Tepp et al. 2011; Aon and Cortassa 2012). According to Kholodenko, Westerhoff, and their coworkers, the sum of the FCC of the metabolic pathway components exceeding one indicates a direct channeling in the pathway (Moreno-Sanchez et al. 2008). On the contrary, in isolated heart mitochondria and permeabilized cardiac fibers the sum of FCC of respiratory chain complexes, ATP synthase, and metabolite carriers, estimated under conditions of respiration activated by ADP, is close to 1, corresponding to a linear metabolic pathway (Moreno-Sanchez et al. 2008; Kuznetsov et al. 1996; Doussiere et al. 1984; Fell and Thomas 1995; Groen et al. 1982). The high efficiency of energy flux control in MI makes this supercomplex a key site for the feedback of metabolic regulation of mitochondrial respiration in cardiac cells (Saks et al. 2012; Tepp et al. 2011).

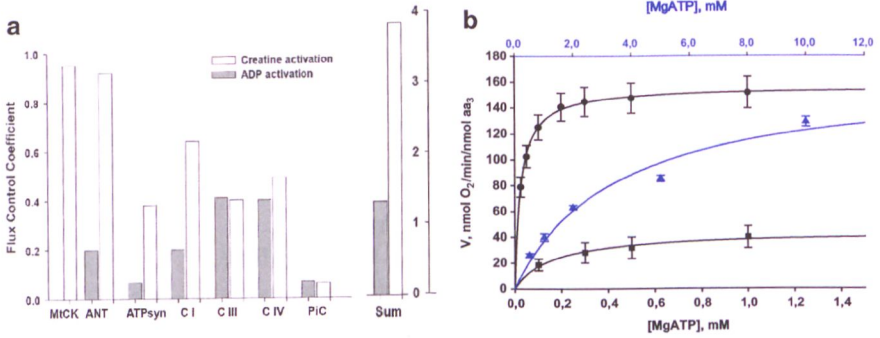
Figure 11.9b depicts the possible role of both Cr and ADP in the control of respiration *in situ*. Extra- and intra-mitochondrial ADP in the regulation of respiration was studied by MgATP titration in the absence or presence of Cr, i.e., activated MtCK (Saks et al. 2012; Guzun et al. 2009; Guzun and Saks 2010; Timohhina et al. 2009). The influence of mitochondrial ADP alone on respiration was estimated by removing extra-mitochondrial ADP through the PEP-PK trapping system mimicking glycolytic ADP consumption. From Fig. 11.9b we can see that stimulation of the extra-mitochondrial ADP producing system by MgATP alone cannot effectively activate respiration. The high apparent  $K_m$  for exogenous MgATP ( $157.8 \pm 40.1 \mu\text{M}$ ) corresponds to the apparent  $K_m$  of myofibrillar ATPase reaction for MgATP. However, when oxidative phosphorylation is stimulated by both extra- and intra-mitochondrial ADP (in the presence of Cr to activate MtCK and MM-CK in myofibrils), the respiration rate increases rapidly up to maximal values and the apparent  $K_m$  for ATP decreases from  $157.8 \pm 40.1 \mu\text{M}$  to  $24.9 \pm 0.8 \mu\text{M}$ . Removal of extra-mitochondrial ADP by PEP-PK provokes an increase of  $K_m$  for MgATP up to  $2.04 \pm 0.10 \text{ mM}$ . These results show that local endogenous ADP in ICEUs is an important regulatory factor of respiration but only in the presence of Cr and activated MtCK. The stimulatory effect of respiration by endogenous ADP is strongly amplified by functional coupling of MtCK with ANT





**Fig. 11.8** Control of mitochondrial respiration by creatine in permeabilized cardiomyocytes. (a) Schematic representations of an oxygraph experiment and of a mitochondrion in a permeabilized cardiac cell, surrounded by cytoskeletal proteins and myofibrils. First, added ATP is hydrolyzed by cellular ATPases and the ADP produced stimulates respiration. Phosphoenolpyruvate (PEP) and pyruvate kinase (PK) continuously trap extra-mitochondrial ADP to regenerate ATP. Stepwise addition of Cr in the presence of ATP stimulates mitochondrial creatine kinase (MtCK) that controls respiration through continuous intra-mitochondrial re-cycling of ADP from ATP. (b) Oxygraph recording of Cr stimulated respiration. This experiment enables the estimation of the apparent affinity of MtCK for Cr. The *left scale* and the *blue trace* indicate the oxygen concentration (nmol  $O_2$  ml<sup>-1</sup>) in the experimental milieu. The *right scale* and the *red trace* denote the rate of oxygen uptake (in nmol  $O_2$  min<sup>-1</sup> nmol<sup>-1</sup> cyt. aa<sub>3</sub>). Adapted from (Guzun et al. 2009) with permission

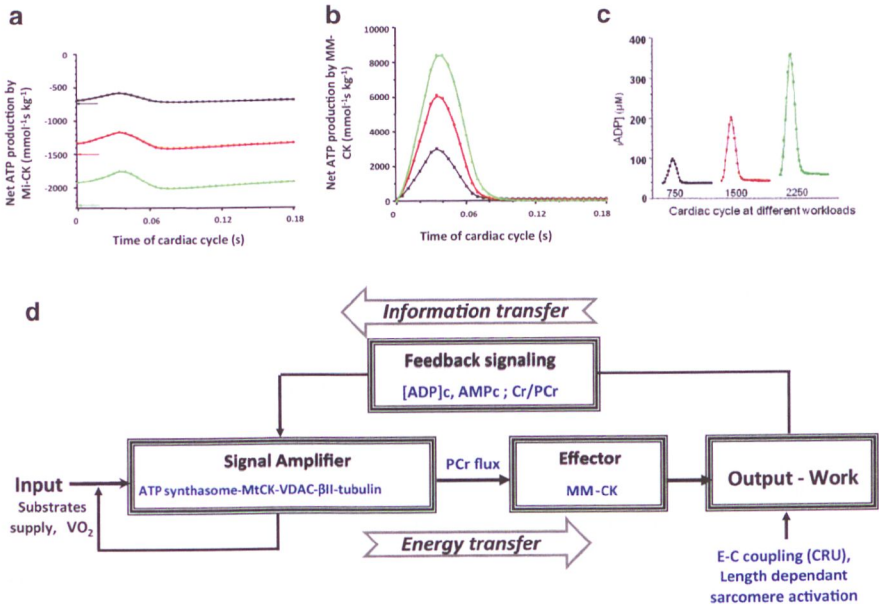
that increases adenine nucleotides recycling within the MI (Saks et al. 2012; Guzun et al. 2009; Timohhina et al. 2009; Jacobus and Saks 1982). The loss of Cr-stimulated respiration in transgenic MtCK-knockout mice confirms the central role of MtCK in respiration regulation (Kay et al. 2000).



**Fig. 11.9** *The energy flux control in permeabilized cardiomyocytes: creatine stimulation of mitochondrial respiration. (a)* Flux control coefficients for MtCK, adenine nucleotide translocase (ANT), ATP synthasome (ATPsyn), respiratory complexes I (C I), III (C III), IV (C IV), and inorganic phosphate carrier (PiC). The right panel shows the sum of flux control coefficients. Reproduced from (Tepp et al. 2011) with permission. **(b)** The role of endogenous ADP produced in MgATPase reactions at different concentrations of MgATP in the regulation of mitochondrial respiration in permeabilized cardiomyocytes under different conditions: (square)—without ADP trapping system (PEP-PK) and in the absence of Cr; (filled circle)—without PEP-PK system but in the presence of 20 mM Cr (i.e., activated MtCK); (triangle)—in the presence of both trapping system for free ADP and 20 mM Cr. Reproduced from (Timohhina et al. 2009) with permission

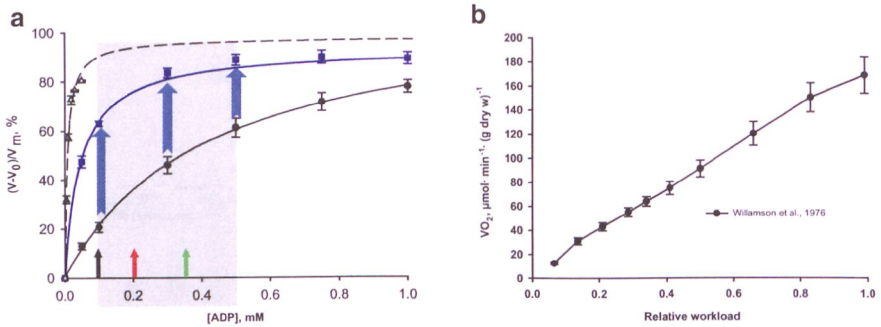
Taken together this information allows explaining the linear relationship existing between oxygen consumption and cardiac work by local metabolic feedback signaling within ICEUs (Saks et al. 2010, 2012; Aliev et al. 2012) (Fig. 11.10). Direct flux determination and mathematical modeling show that not more than 10 % of free energy is transported out of mitochondria by ATP flux needed to equilibrate the information-carrying flux of ADP into mitochondria. According to this model, ADP released from actomyosin cross-bridges stimulates the local MM-CK reaction in the myofibrillar space within ICEUs while at the same time forms a concentration gradient towards mitochondria (Fig. 11.10a–c) (Dos Santos et al. 2000; Aliev et al. 2012; Aliev and Saks 1997; Vendelin et al. 2000). The amplitude of displacement of MM-CK from equilibrium, as well as cyclic changes in ADP, is proportionally increased with workload (Fig. 11.10b, c). The rephosphorylation of ADP in the MM-CK reaction increases locally the Cr/PCr ratio that is transferred towards MtCK via the CK/PCr shuttle. Regulation of VDAC permeability by  $\beta$ II tubulin is a key element mediating the linear response of mitochondrial respiration to local signaling within ICEUs. When MOM is permeable, as in isolated mitochondria, modulation of respiration is impossible because of saturating ADP concentrations used under these conditions. The latter exceeds manifold the apparent affinity of oxidative phosphorylation for free ADP ( $K_m^{app}ADP = 7.9 \pm 1.6 \mu M$ ), even in diastolic phase (about 40  $\mu M$ ) (Fig. 11.11a). On the contrary, when ADP diffusion is restricted at the level of





**Fig. 11.10** Mechanisms of regulation of mitochondrial respiration controlled by MtCK and of energy fluxes in cardiac muscle cells. (a–c) Results from a mathematical model of cardiac energy metabolism (Vendelin-Aliev-Saks-Dos Santos model). (a,b) Calculated net PCr production rates in nonequilibrium steady state MtCK reaction (a) and cyclic changes in rates of ATP regeneration in nonequilibrium myofibrillar MM-CK reaction (b) during contraction cycles at different workloads corresponding to oscillations of [ADP]<sub>c</sub> indicated in Fig. 11.11. (c) Mathematically modeled oscillations of ADP concentrations in the core of myofibrils over cardiac cycle at workloads equivalent to 750 (black), 1,500 (red) and 2,250 (green)  $\mu\text{mol ATP s}^{-1} \text{kg}^{-1}$ . According to this model, the ATP cyclically produced during contractions (b) is associated with cyclical oscillations of ADP and Pi concentrations in myofibrils (c) and subsequent PCr production in the MtCK reaction (a). Reproduced from (Dos Santos et al. 2000; Aliev et al. 2012; Aliev and Saks 1997; Vendelin et al. 2000) with permission. (d) Schematic representation of feedback metabolic signaling in regulation of energy metabolism within ICEUs in cardiac cells. Due to the nonequilibrium MtCK and cyclic MM-CK reactions, intracellular ATP utilization (output) and mitochondrial ATP regeneration (input) are linked via cyclic fluctuations of cytosolic ADP and Cr/PCr. See the text for explanation

MOM, as in mitochondria in situ, the apparent  $K_m$  for free ADP increases to about  $370.75 \pm 30.57 \mu\text{M}$  and the respiration rate becomes almost linearly dependent on local ADP concentration. Under these conditions, the initial respiratory rate can be approximated by its linear dependence on ADP within the range of values corresponding to the increase in workload (Fig. 11.11b) (Guzun et al. 2009; Timohhina et al. 2009). Thus, cyclic changes in local ADP concentrations within the myofibrillar space of ICEUs become an effective regulatory signal due to (1) the nonequilibrium state of CK reactions, (2) the restricted VDAC permeability to metabolites elicited by association with  $\beta$ II tubulin, and (3) the presence of



**Fig. 11.11** The role of restriction of ADP diffusion in the regulation of mitochondrial respiration. (a) Kinetic analysis of ADP-activated respiration. The ADP concentrations corresponding to mathematically modeled fluctuations of ADP by Michaelis–Menten graph representation with colored small arrows (black, red and green), contained in the area of physiological cytosolic ADP concentration (indicated by a gray box). When MOM is permeable, as in isolated mitochondria ( $\Delta$ ,  $K_m^{\text{app}}\text{ADP}$ — $7.9 \pm 1.6 \mu\text{M}$ ), the regulation of respiration is impossible because of a saturated ADP concentration for the minimal workload. When the ADP diffusion is restricted at the level of MOM, as in mitochondria in permeabilized cardiomyocytes (circle,  $K_m^{\text{app}}\text{ADP}$ — $370.75 \pm 30.57 \mu\text{M}$ ), the respiration rates become linearly dependant on ADP concentrations, in fact also on heart workloads in accordance with the Frank–Starling law (b). This linear dependence under physiological conditions can be amplified by creatine (see large blue arrows in a) in the presence of activated MtCK (Square,  $K_m^{\text{app}}\text{ADP}$ — $50.24 \pm 7.98 \mu\text{M}$ ). Reproduced from (Guzun et al. 2009) with permission. (b) The metabolic aspect of the Frank–Starling’s law of the heart is expressed by linear dependence between the increase of left ventricular end-diastolic volume and the increase of respiration rates in the absence of measurable changes in the intracellular ATP and PCr content. Reproduced from (Saks et al. 2006c) with permission

Cr. When these conditions are fulfilled, activation of the coupled MtCK within MI by Cr induces ADP/ATP recycling and increases respiration rate, thus amplifying the effect of cytoplasmic ADP; under these conditions, the apparent  $K_m$  for ADP becomes equal to  $50.24 \pm 7.98 \mu\text{M}$  (Fig. 11.11a). These data suggest that modulation of respiration by local changes in ADP concentration, under condition of restriction of adenine nucleotide diffusion across mitochondrial membranes, is mediated by the structural organization of the MI. The MtCK reaction amplifies the ADP signal due to its functional coupling with ATP Synthasome (Fig. 11.7), thus increasing the steady-state rate of adenine nucleotides cycling in mitochondria and the rate of respiration. The coupled reactions of muscle type MM-CK in myofibrils and MtCK in mitochondria perform under nonequilibrium conditions and proceed in opposite directions (Fig. 11.10a–c) (Saks et al. 2012; Guzun et al. 2009; Guzun and Saks 2010; Timohhina et al. 2009). This mode of function results in separation of energy fluxes (mass and energy transfer by PCr) and signaling (information transfer by oscillations of cytosolic ADP concentrations, Pi and PCr/Cr ratio) that is amplified within the MI. As a result, reactions catalyzed by different isoforms of compartmentalized CK tend to maintain the intracellular metabolic stability. The separation of energy and information transfer is illustrated



by the scheme depicted in Fig. 11.10d. This scheme shows feedback regulation of respiration *in vivo* according to Norbert Wiener's cybernetic principles (Saks et al. 2012; Guzun and Saks 2010): the usage of ATP (or release of free energy of ATP hydrolysis,  $\Delta G_{\text{ATP}}$ , to perform work, marked as output) and ATP regeneration (or extraction of  $\Delta G_{\text{ATP}}$  from substrates by oxidative phosphorylation, denoted as input) are interconnected via the feedback signaling through oscillations of cytoplasmic concentrations of ADP, AMP, Pi, and Cr/PCr amplified within MI. In this framework, the role of  $\beta$ II tubulin in association with MOM in cardiomyocytes would be to induce the linear response of mitochondrial respiration to workload-dependent metabolic signals. This elegant feedback mechanism of regulation of respiration on a beat-to-beat basis ensures metabolic stability necessary for normal heart function and explains well the metabolic aspect of the Frank–Starling's law of the heart (Saks 2007; Saks et al. 2006a, 2012). Importantly, recycling of adenine nucleotides within MI when coupled to PCr production significantly decreases ROS levels ensuring maximal efficiency of free energy transduction in mitochondria while inhibiting permeability transition pore opening, thus protecting the heart under stress conditions (Schlattner et al. 2006b; Meyer et al. 2006).

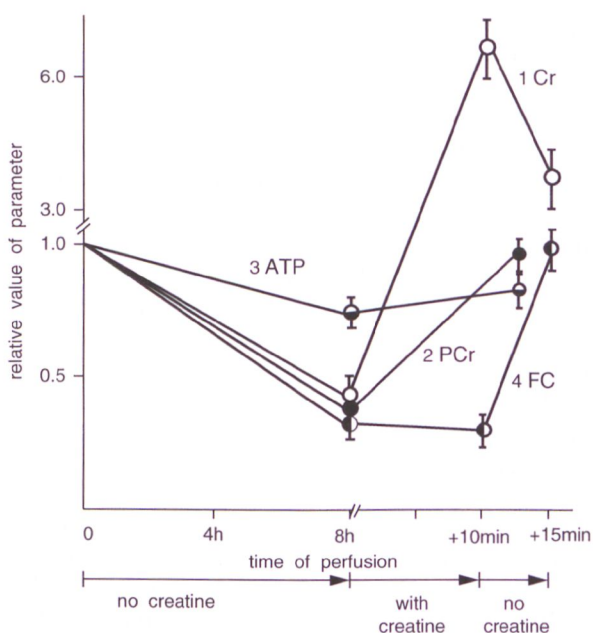
While the mechanisms described above represent local signaling within ICEUs, important mechanisms of synchronization of mitochondrial activity between ICEUs and their integration into structurally and functionally organized cellular systems are described by Cortassa and Aon in Chap. 5. The role of  $\text{Ca}^{2+}$  cycle in maintaining high respiratory activity of mitochondria within ICEUs has been described by Balaban's group and studied by mathematical modeling by Cortassa et al. (2009).

#### ***11.4.4 Intracellular Creatine Concentration as a Regulatory Factor in Heart Energetics***

Many experimental and clinical studies have shown that intracellular Cr concentration is an important factor, determining the efficiency of intracellular energy transfer in heart cells (Saks et al. 1978, 2012; Wyss and Kaddurah-Daouk 2000; Nascimben et al. 1996). The results of an earlier work of ours published more than 30 years ago are reproduced in Fig. 11.12. This experiment shows that removal of Cr from the frog heart cells results in decreased PCr content and diminished contractile force; all parameters return to their initial value after restoration of Cr content (Saks et al. 1978). Similar results were recently reported by (Nabuurs et al. 2013) by assessing morphological, metabolic, and functional consequences of systemic Cr depletion in skeletal muscle. These data were obtained in a mouse model deficient in *L*-arginine:glycine amidino transferase ( $\text{AGAT}^{-/-}$ ) which catalyzes the first step of Cr biosynthesis. In this work, systemic Cr depletion resulted in mitochondrial dysfunction and intracellular energy deficiency, as well as structural and physiological abnormalities. *In vivo* magnetic resonance

**Fig. 11.12** *The role of Creatine in the regulation of contraction in frog heart.*

After 8 h of perfusion without creatine, frog heart strips assume a hypodynamic state with decreased contractile force (FC) and lowered Cr and PCr levels. Addition of 20 mM Cr to the perfusate restored to normal the values of all these variables. Reproduced with permission from (Saks et al. 1978)



spectroscopy showed a near-complete absence of Cr and PCr in resting hind limb muscle of  $AGAT^{-/-}$  mice. Compared to wild type, the inorganic phosphate/ $\beta$ -ATP ratio was increased fourfold, while ATP levels were reduced to nearly half and overall mitochondrial content was increased. The Cr-deficient  $AGAT^{-/-}$  mice presented with significantly reduced grip strength and suffered from severe muscle atrophy. Oral Cr administration led to rapid accumulation in skeletal muscle (faster than in brain) and reversed all muscle abnormalities revealing that the condition of the  $AGAT^{-/-}$  mice can be switched between Cr-deficient and normal simply by dietary manipulation. The consequences of AGAT deficiency were more pronounced than those of muscle-specific CK deficiency (Nabuurs et al. 2013), which suggests a multifaceted involvement of Cr in addition to its role in the PCr-CK system and in muscle energy homeostasis, as, e.g., by direct effects on biomembranes (Tokarska-Schlattner et al. 2012). It was also shown by the group of Stefan Neubauer in Oxford that a moderate elevation of total Cr levels in the heart by approximately 50 % in transgenic mice overexpressing the Cr transporter (CRT) conveyed significant protection and improved recovery of the hearts upon experimental induction of ischemia/reperfusion (Lygate et al. 2012). In one of their most important work the Neubauer's group has shown that a decrease of PCr content in the heart of patients with dilated cardiomyopathy is accompanied with significantly increased mortality rates (Neubauer 2007).

The role of altered phosphotransfer pathways in heart pathology of animal models, as well as human patients, is well documented and has been described in



a number of reviews (Ingwall and Weiss 2004; Ingwall 2006; Ventura-Clapier et al. 2002, 2004). Most recently, two younger Chinese patients with acute myocardial infarction and presenting with muscle MM-CK deficiency have been diagnosed with somatic mutations in the M-CK gene. These mutants at amino acid E79 prevent correct folding and dimerization of M-CK. In parallel, correct targeting of the enzyme to subcellular structures is hampered and enzymatic CK activity dramatically lowered (Wu et al. 2013). These data with human cardiac infarction patients have shown that active dimeric MM-CK together with its substrates Cr and PCr are important for normal heart function.

Thus, the current opinion, supported by a host of data derived from different experimental approaches and provided by a number of different independent laboratories, is that Cr and PCr together with microcompartmentalized CK isoforms are physiologically essential for normal body function, specifically for optimal performance of skeletal and heart muscle, brain, neuronal cells, skin, retina and auditory cell, spermatozoa, and other cells of intermittent high-energy requirements (Wallimann et al. 1992, 2011). This fundamental hypothesis is strongly supported by the more or less severe phenotypes observed in double and single CK isoenzyme knockout mice, respectively (Steeghs et al. 1997; Streijger et al. 2005; Heerschap et al. 2007), as well as by the phenotypes of AGAT and GAMT knockout mice, presenting with disturbed energy metabolism body weight control, hampered fertility, muscular dystrophy, and cognitive and behavioral impairment, etc. (Nabuurs et al. 2013; Schmidt et al. 2004; ten Hove et al. 2005; Torremans et al. 2005).

In a most recent, provocative publication, entitled “Life without creatine”, Lygate and colleagues purport that the phenotype of the GAMT knockout mouse was basically “normal”. Specifically, they do not find a skeletal or cardiac muscle phenotype (Lygate et al. 2013). This contradicts the phenotype of the same transgenic mouse described earlier (Schmidt et al. 2004; ten Hove et al. 2005; Kan et al. 2005). Most importantly it is in contrast to the AGAT knockout creatine deficiency mouse (Nabuurs et al. 2013). This latter AGAT knockout mouse, in contrast to the GAMT knockout mouse, does not synthesize guanidine acetate (GAA), which in the GAMT knockout skeletal muscle was shown to be phosphorylated by CK to form an alternative energy-rich phosphagen, phospho-GAA, which still can be utilized as high-energy phosphagen, albeit at lower efficiency (Heerschap et al. 2007). Thus, it will be most important to reevaluate cardiac energy metabolism and heart phenotype in the GAMT knockout mouse to completely rule out any compensatory effects of the high concentrations of phospho-GAA accumulated in these knockout mice and to rule out a still possible contribution of phospho-GAA as an still alternative energy source. Corresponding experiments with the pure creatine deficiency AGAT knockout, presenting with a rather severe phenotype that is reversible by simple creatine supplementation (Nabuurs et al. 2013), are warranted and should provide some answers to these pending questions. To get a physiologically more meaningful answer to the true function of CK in heart it will be paramount to stress the heart to maximal performance and work output, where one would expect to see the true effects of creatine deficiency also in this organ.

In conclusion, until the enigma of the results provided by (Lygate et al. 2013) (see above) is solved, all available data still indicate that the CK system together with PCr and Cr is central to the regulatory mechanisms of metabolic and energy fluxes in those cells under intermitantly fluctuating high-energy requirements, including the heart (Taegtmeyer and Ingwall 2013).

## **11.5 The Signaling Network of AMP-Activated Protein Kinase (AMPK) in the Heart**

### ***11.5.1 Protein Kinase Signaling Networks in Metabolic Control of Cardiac Function***

Metabolic cycles as described before provide immediate metabolic feedback for changes in energy input (nutrient supply) and energy output (workload). They are particularly important in the heart, an organ that maintains a high degree of metabolic stability and a particularly well-controlled energy homeostasis. An additional layer of regulation, which ascertains this metabolic stability, is achieved by information transfer via protein kinase signaling. All major protein kinase pathways were shown to play important roles in the heart, controlling contraction force, contractility, and heart rate in particular during cardiac development, under prolonged strong stimulation, and under emerging pathological conditions.

The possibly best studied example is the cyclic adenosine nucleotide (cAMP)-dependent protein kinase A (PKA) (Taylor et al. 2008), together with its homologous cGMP-dependent protein kinase G (PKG) (Takimoto 2012). Their control of cardiac contraction strength, ion fluxes, and hypertrophy relies on a precise spatiotemporal regulation of substrate phosphorylation. In case of PKA, A-kinase anchoring proteins (AKAPs) and cyclic nucleotide phosphodiesterases (PDEs) play a major role in this spatiotemporal organization and the occurrence of cAMP microdomains (Edwards et al. 2012; Mika et al. 2012; Diviani et al. 2013). This emphasizes the importance of cellular localization and organization for protein kinase-mediated information fluxes, as already outlined above for cardiac CK isoenzymes.

Also some other protein kinase signaling pathways have to be considered as relevant for cardiac metabolism. Protein kinase B (PKB or Akt) is an essential component of the growth response of an organism to nutritional input. In the heart, it participates in the regulation of myocyte growth under physiological conditions (Walsh 2006; Hers et al. 2011). PKC isoforms regulate cardiac contraction and hypertrophic responses, as well as other signaling pathways in more pathological situations such as ischemia and reperfusion injuries (Steinberg 2012). While calcium-regulated PKD is a more recent addition to the kinome, less well studied in respect to the cardiovascular system (Avkiran et al. 2008), members of the



mitogen-activated protein kinase (MAPK) family are prominent regulators of cardiac function with both protective and detrimental effects (Rose et al. 2010).

The protein kinase most relevant in the context of metabolic stability and energy homeostasis, however, is AMP-activated protein kinase (AMPK). It has often been described as a major “signaling hub,” “fuel gauge,” “metabolic sensor,” or “metabolic master switch” because it plays a central role in sensing and regulating energy homeostasis at the cellular, organ, and whole-body level (Winder and Hardie 1999; Hardie and Carling 1997). Activation of AMPK is triggered by a diverse array of signals linked to limited energy availability in physiological and pathological situations, including extracellular (e.g., hormones, cytokines, nutrients) and intracellular stimuli (e.g., AMP, ADP) (Hardie et al. 2012a). Activation involves covalent phosphorylations and allosteric binding of AMP or ADP that cooperate in a complex manner. In general, these regulations are coordinated to activate AMPK in situations of energy deficits and aim at compensating ATP loss via accelerated catabolism and inhibited anabolism. However, pleiotropic control exerted by AMPK affects not only metabolic pathways but also other physiological functions like cell growth and proliferation, cell polarity and motility, apoptosis, autophagy, and central appetite control by regulating enzyme activities and gene transcription. This has made the kinase also a prime pharmacological target for treating metabolic disorders or possibly also cancer (Hardie 2007; Zhang et al. 2009; Finckenberg and Mervaala 2010; Inoki et al. 2012; Srivastava et al. 2012).

Although earlier work on AMPK mainly focused on tissues like liver and skeletal muscle, more recent research has revealed novel molecular mechanisms of AMPK regulation and downstream action that are relevant also to cardiovascular function. Activation of the AMPK pathway plays a particularly important role in the myocardial response to pathological stimuli like ischemia–reperfusion (Kudo et al. 1995; Russell et al. 2004), pressure overload (Tian et al. 2001; Kim et al. 2012), or heart failure (Sasaki et al. 2009). Pharmacological activation of AMPK also holds promise as a therapeutic strategy for treating cardiovascular diseases (e.g., Sasaki et al. 2009; Calvert et al. 2008; Shinmura et al. 2007). The following paragraphs will briefly summarize the key elements of AMPK signaling with an emphasis on metabolic regulation in the heart. For more complete reviews of this issue, the reader is referred to some excellent recent overviews dedicated to general AMPK signaling (Hardie et al. 2012a; Inoki et al. 2012; Carling et al. 2012; Oakhill et al. 2012) or AMPK functions in the heart (Zaha and Young 2012; Harada et al. 2012; Ahn et al. 2012; Horman et al. 2012) and other organs [see e.g., a recent review series in *Mol Cell Endocrinol* (Steinberg 2013)]. Thus, AMPK signaling may constitute a fourth module for a systems description of the cardiac metabolic network.

### ***11.5.2 Network Elements: AMPK Isoforms and Their Distribution in Cells and Tissues***

AMPK is an evolutionary conserved and ubiquitously expressed serine/threonine kinase that presents complex structural and functional features. Structurally, it occurs in vertebrates as an obligatory heterotrimeric complex composed of one catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ). As a first layer of complexity, all subunits exist in form of different isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma 1$ ,  $\gamma 2$ , and  $\gamma 3$ ) and splice variants (of  $\gamma 2$  and  $\gamma 3$ ), generating multiple heterotrimeric complexes. The precise physiological significance of these isoforms is not yet entirely clear. However, there is some evidence that they determine intracellular distribution, protein recognition, or tissue-specific functions of AMPK, all of which could provide selectivity for specific subsets of substrates within the ever increasing list of AMPK substrates (Hardie et al. 2012a, b; Carling et al. 2012).

#### **11.5.2.1 Subcellular Localization**

The subcellular distribution and recruitment of AMPK to specific sites are likely an important factor for its signaling function, but so far only few details are known, in particular in heart. AMPK is generally observed as a soluble complex with diffuse cytosolic localization, but at least  $\alpha 2$ -containing complexes in their activated form can translocate into the nucleus to phosphorylate important substrates (e.g., transcription factors, histones, histone deacetylases) as seen, e.g., after exercise in skeletal muscle (McGee et al. 2003, 2008; Suzuki et al. 2007; McGee and Hargreaves 2008). Minor but important portions of AMPK may associate with cellular structures like specific membranes, where processes are regulated by AMPK (e.g., ion channel activity, cell polarity, or cell junction formation) (Forcet and Billaud 2007; Andersen and Rasmussen 2012; Nakano and Takashima 2012). Myristoylation of the AMPK  $\beta$ -subunit can localize the kinase complex to membranes and increases its activability, possibly favoring activation of membrane-bound complexes (Suzuki et al. 2007; Oakhill et al. 2010).

AMPK may also be recruited into specific complexes via interaction with its upstream kinases, downstream substrates, or more general scaffolding proteins. However, the AMPK interactome is only partially known so far from some targeted and non-biased interaction studies conducted by us and others (e.g., Ewing et al. 2007; Moreno et al. 2009; Behrends et al. 2010; Klaus et al. 2012), and more research is needed on this issue, in particular in the heart. AMPK interaction with LKB1, its major upstream kinase in the heart, could localize AMPK to places of LKB1 localization, including the mitochondrial surface or E-cadherin in adherens junctions (Sebbagh et al. 2009). Scaffolding proteins can provide specificity in cell signaling by isolating activated kinases from bulk signaling and directing the information flow into specific pathways. In heart, for example, AMPK competes with p38 MAPK for binding to the scaffolding protein



TAK-1-binding protein-1, thus blunting p38 activation during ischemia (Li et al. 2005). Mitochondrial VDAC may represent yet another anchor protein recruiting AMPK to this organelle (Strogolova et al. 2012). There is also some evidence that AMPK subunit isoforms determine specific protein interactions. The  $\beta$ -subunit may in some cases confer substrate specificity, as seen with the yeast and plant orthologues (Vincent and Carlson 1999; Polge et al. 2008), but also putative mammalian AMPK interactors (IntAct database, (Kerrien et al. 2012)). We recently found the  $\beta$ 2-isoform interacting with Mu- and Pi-type glutathione transferases (GSTs) to favor glutathionylation of the  $\alpha$ -subunit (Klaus et al. 2013). However, in case of fumarate hydratase (FH), we identified a specific interaction with  $\alpha$ 2-containing AMPK complexes (Klaus et al. 2012).

### 11.5.2.2 Expression Patterns

AMPK isoforms also show some differences in their tissue- and developmental-specific expression patterns, also in heart, although the physiological significance is still uncertain. While the  $\alpha$ 1 $\beta$ 1 $\gamma$ 1 complex is probably the most abundant in most cell types, differences seem to occur in the amount of additional isoforms like  $\alpha$ 2- and  $\gamma$ 3 in skeletal muscle or  $\beta$ 2 and a specific intermediate length  $\gamma$ 2 splice variant ( $\gamma$ 2-3B) in the heart (Stapleton et al. 1996; Thornton et al. 1998; Li et al. 2006; Pinter et al. 2012). There are also pathological and developmental changes in cardiac AMPK expression. The  $\alpha$ 2,  $\beta$ 2, and  $\gamma$ 2 isoforms are all upregulated by pressure overload or heart failure in rodents, although in patients rather the content of  $\alpha$ 1,  $\beta$ 1, and  $\gamma$ 2 (an intermediate form) increases with different forms of cardiomyopathy (Tian et al. 2001; Kim et al. 2012). During embryonic development in rodents,  $\gamma$ 1 increases while high levels of  $\gamma$ 3 disappear, and the embryonically predominant full-length  $\gamma$ 2 form is replaced by  $\gamma$ 2-3B in heart, but by short  $\gamma$ 2b in other tissues (Pinter et al. 2012). These developmental and tissue particularities may also explain why  $\gamma$ 2 gene mutations in the CBS domains cause hereditary hypertrophic cardiomyopathy (see below) but no other pathological symptoms. Full-length  $\gamma$ 2 and  $\gamma$ 2-3B share an N-terminal domain with unknown function that could localize the AMPK complex to specific compartments or signaling pathways (Pinter et al. 2012). Total AMPK activity increases after birth, contributing to the switch to predominant use of fatty acids (Makinde et al. 1997). AMPK levels may also be determined by ubiquitin-dependent mechanisms (Qi et al. 2008; Moreno et al. 2010), but its role in the heart is not known.

## 11.5.3 Network Elements: Molecular Structure of AMPK

Given the interest in AMPK as a putative drug target in metabolic diseases, recent years have seen intense efforts to elucidate the molecular structure of AMPK.

By solving several X-ray structures for AMPK domains and truncated core complexes, the topology of the heterotrimer, the conserved global fold of large parts of the subunits, and the putative activation mechanisms could be deduced (Townley and Shapiro 2007; Amodeo et al. 2007; Xiao et al. 2007, 2011; Chen et al. 2009; Oakhill et al. 2011). However, a high-resolution structure of full-length heterotrimeric complex in both active and inactive states is still lacking. The so far most complete X-ray structure covers most of the  $\alpha$ 1-subunit except a C-terminal linker region (although not all of the sequence present is well resolved in the electron density map), the C-terminal domain of the  $\beta$ 2-subunit, and the entire  $\gamma$ 1-subunit (Xiao et al. 2007, 2011).

#### 11.5.3.1 $\alpha$ -Subunit

The catalytic  $\alpha$ -subunit contains an N-terminal serine-threonine kinase domain with an activation loop carrying the critical Thr172 residue. Phosphorylation of this residue by upstream kinases is essential for AMPK activation and often used as an indicator for AMPK activity (Hawley et al. 1996). The C-terminal  $\alpha$ -domain carries the motif interacting with the  $\beta$ -subunit and further structural determinants that are involved in AMPK activation. These include an autoinhibitory domain (AID) and loop(s) contacting the  $\gamma$ -subunit (called  $\alpha$ -hook or  $\alpha$ -RIM1 and  $\alpha$ -RIM2) (Xiao et al. 2007; Chen et al. 2009, 2013a; Pang et al. 2007). Their exact roles are, however, disputed and further structural studies will be necessary to delineate their function in autoinhibition and  $\alpha$ - $\gamma$  communication. Earlier in vitro studies suggested that the  $\alpha$ 2 subunit has a higher sensitivity to allosteric activation by AMP (Salt et al. 1998).

#### 11.5.3.2 $\beta$ -Subunit

The regulatory  $\beta$ -subunit is often described as a scaffold for  $\alpha$ - and  $\gamma$ -subunits, a function that indeed is provided by the C-terminal domain. The N-terminal domain whose structure is not entirely resolved at molecular resolution carries additional regulatory elements. A glycogen-binding domain (GBD) seems to provide regulation of AMPK by glucose  $\alpha$ 1-6-branched glycogen that inhibits AMPK activation (Polekhina et al. 2003; McBride et al. 2009). The N-terminal  $\beta$ -domain may also be involved in protein interactions of AMPK (see above).

#### 11.5.3.3 $\gamma$ -Subunit

While  $\alpha$ - and  $\beta$ -isoforms are very homologous,  $\gamma$ -subunits and their splice variants differ in length. They all share the C-terminal part that consists of four cystathionine  $\beta$  synthetase (CBS) domains that are arranged in tandem in so-called Bateman domains (Bateman 1997). The symmetrical structure of this



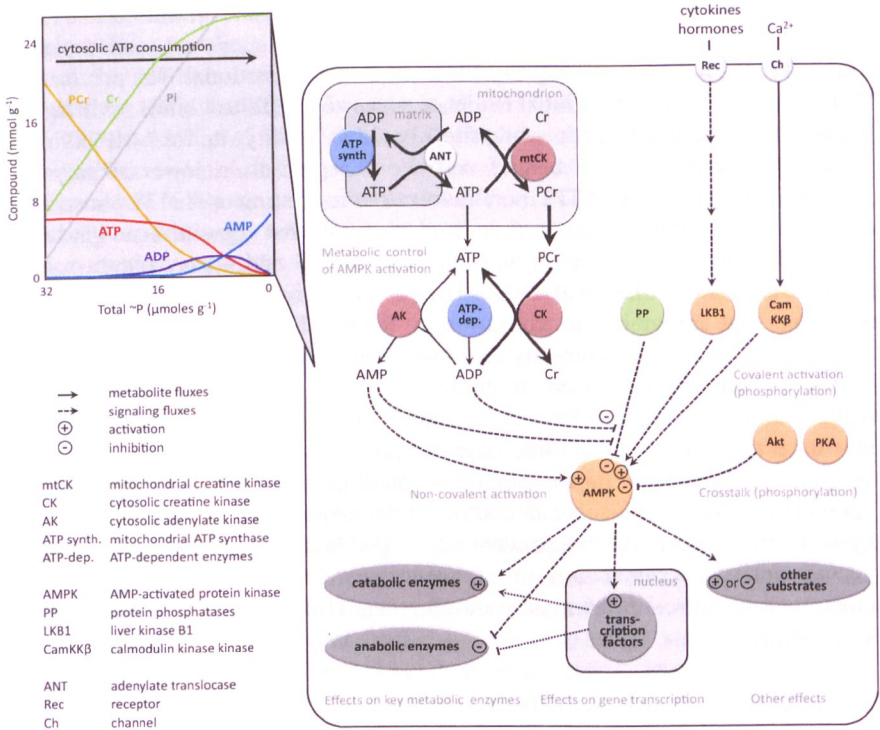
domain theoretically provides four binding sites for adenylates [referred to as sites 1–4 (Kemp et al. 2007; Hardie et al. 2011)], but only sites 1, 3, and 4 can be occupied in the mammalian enzyme, while site 2 is nonfunctional. The precise role of these sites is still unclear. Initial evidence suggested that site 4 binds AMP tightly in a non-exchangeable manner, while site 1 is a high-affinity site for AMP involved in allosteric activation (see below) and site 3 represents a lower affinity site (binding AMP, ADP, and ATP) more involved in regulating  $\alpha$ -Thr172 phosphorylation (Xiao et al. 2007). A more recent study suggests that also site 4 can bind ATP (and causes site 3 to be empty) and that both sites 3 and 4 may play a role in allosteric activation (Chen et al. 2012). The  $\gamma$ 2- and  $\gamma$ 3-isoforms contain N-terminal extensions that are subject to truncation by RNA splicing and whose molecular structure and function are currently unknown. Mutations in the CBS domains of the AMPK  $\gamma$ 2 subunit, expressed at particularly high levels in heart, cause the Wolff–Parkinson–White (WPW) syndrome, a hereditary cardiomyopathy of varying severity, involving cardiac hypertrophy, contractile dysfunction, and arrhythmias. Mutations impair adenylate binding and thus AMPK activation (Scott et al. 2004; Burwinkel et al. 2005), but the major cause for the cardiomyopathy is the increased AMP-independent basal AMPK activity. This leads to higher glucose uptake, accumulation of glycogen in cardiac myocytes, and finally impairment of normal heart muscle development (Burdwinkel et al. 2005; Davies et al. 2006).

### ***11.5.4 Network Connectivity: AMPK Input Signals and Upstream Regulation***

AMPK integrates various intra- and extracellular signals and maintains cross talk with other signaling pathways, all related to the cellular energy and nutrient state. This makes the kinase a central signaling hub in sensing and regulating cellular energetics and ATP-dependent functions. Indeed, most recent research revealed that AMPK activation is much more complex than initially anticipated and depends on multiple covalent modifications and allosteric effectors (Fig. 11.13). AMPK regulation also evolved from a more simple state as, e.g., in the yeast AMPK homologues that lack allosteric activation by AMP (see below) to the more complex regulation present in mammals.

#### **11.5.4.1 Covalent Regulation by Phosphorylation**

The phosphorylation state at  $\alpha$ -Thr172 defines the primary activation of AMPK. This is determined by the balance of different upstream kinases and phosphatases. There are potentially three mammalian AMPK upstream kinases, with the major one in most cell types, including heart, being Liver Kinase B1 (LKB1, also called



**Fig. 11.13 AMPK signaling.** Large scheme: Activation of AMPK by intra- and extracellular metabolic and endocrine signals and major fields of downstream signaling. Activation of AMPK is determined by upstream kinases (covalent activation by LKB1, CamKKβ, inhibition by Akt and PKA) and phosphatases. They mediate mainly extracellular signals carrying, e.g., information on the energy and nutrient state of the cellular environment and the entire organism (endocrine signals). Covalent activation also depends on some intracellular parameters (Ca<sup>2+</sup>, possibly also ROS/RNS). As a second layer of regulation, AMPK is activated by ADP and in particular AMP (allosteric regulation), both acting as second messengers of cellular energy stress. This signaling is linked to conversion of nucleotides via the adenylate kinase (AK) and creatine kinase (CK) reactions. Activated AMPK compensates for ATP loss by accelerating catabolism, inhibiting anabolism, and further effects on cell motility, growth, proliferation, and others, via regulation of key enzymes and transcription factors. For further details see text. Insert: Connection of AMPK signaling and phosphotransfer reactions (CK and AK) (Neumann et al. 2003). Global cellular concentration changes of phosphocreatine [PCr] and adenine nucleotides ([ATP], [ADP] and [AMP]), inorganic phosphate [Pi], and creatine [Cr], calculated from the reactions of CK, AK, and a generalized ATPase at decreasing “high-energy” phosphates (corresponding to a transition from rest to high work-load). Note that with “high-energy” phosphate consumption, [ATP] remains constant until more than 80 % of the PCr pool is consumed. Only then, there is a transient increase in [ADP] and finally [AMP] starts to rise dramatically. The exponential rise in [AMP] makes this nucleotide an ideal second messenger for a lowered cellular energy state. This simplified model assumes that the CK and AK reactions work at equilibrium (which is unlikely to be true in vivo) and does not account for specific subcellular localizations of CK and AK



STK11). LKB1 is upstream of an entire family of 12 other AMPK-related kinases in the human kinome, and like AMPK forms heterotrimers with two accessory subunits, STRAD $\alpha/\beta$  and MO25 $\alpha/\beta$  (Hawley et al. 2003; Woods et al. 2003). LKB1 has originally been identified as a tumor suppressor whose inactivating mutations lead to the Peutz–Jeghers syndrome, an inherited susceptibility to different human cancers (Alessi et al. 2006). However, LKB1 seems to mostly exhibit constitutive activity and may thus not be the limiting step in AMPK activation. An alternative upstream kinase much less expressed in heart is Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  $\beta$  (CamKK $\beta$ ) that mediates Ca<sup>2+</sup>-dependent AMPK activation (Hawley et al. 2005; Hurley et al. 2005; Woods et al. 2005). Although such CamKK $\beta$ -mediated AMPK activation might anticipate an increasing energy turnover that accompanies a rise in cytosolic Ca<sup>2+</sup> during muscle contraction, its role in the heart is not well understood. More recently, the transforming growth factor- $\beta$ -activated kinase-1 (TAK1) that phosphorylates the yeast AMPK homologue Snf1 was proposed as an AMPK upstream kinase (Momcilovic et al. 2006; Xie et al. 2006a). Although TAK-1 is present in heart, it is not activated during ischemia and it is unclear whether it acts via direct AMPK phosphorylation (Xie et al. 2006a).

Protein phosphatases are possibly the more critical parameter governing the  $\alpha$ -Thr172 phosphorylation state, and this may also apply to the heart. However, their identity and regulatory role *in vivo* remain to be confirmed. Both seem to depend on cell type and/or stimulus. Different phosphatases can act on AMPK, including PP1, PP2A, and PP2C *in vitro* (Davies et al. 1995), as well as PP1-R6 in MIN6 beta cells (Garcia-Haro et al. 2010) and metal-dependent phosphatase PPM1E/F in HEK-293 cells *in vivo* (Voss et al. 2011). In heart and endothelial cells, expression levels PP2C and 2A, respectively, correlate with AMPK activation (Wang and Unger 2005; Wu et al. 2007).

The  $\alpha$ -Thr172 phosphorylation state is further negatively controlled by hierarchical phosphorylation at other sites in the AMPK heterotrimer. Phosphorylation at  $\alpha$ 1-Ser485 ( $\alpha$ 2-Ser491) by PKA or at  $\alpha$ -Ser173 by PKB/Akt reduces  $\alpha$ -Thr172 phosphorylation (Hurley et al. 2006; Horman et al. 2006; Djouder et al. 2010). Further phosphorylation sites were identified in both  $\alpha$ - and  $\beta$ -subunits, many of them targeted by autophosphorylation, but their functional role remains uncertain.

#### 11.5.4.2 Non-Covalent Allosteric Regulation by AMP and ADP

The activation of AMPK by low cellular energy state is triggered by increased concentrations of AMP and, as discovered more recently, also of ADP, since the kinase can sense AMP/ATP and ADP/ATP concentration ratios (Xiao et al. 2011; Oakhill et al. 2011). In many cell types and in particular in heart, breakdown of ATP to ADP at the onset of high workload or cellular stress has only minor immediate effects on ATP levels. Due to the energy buffer and transfer function of the Cr/CK system (see above), global and local ATP is rapidly replenished (Wallimann et al. 2011; Neumann et al. 2003). Thus, ATP is not a very suitable signal for

indicating developing energy deficits. However, minor decreases in ATP levels lead to more pronounced relative increases in free ADP and even more in AMP due to the adenylate kinase (AK) reaction (Fig. 11.13). Under these conditions, AK uses two ADP to regenerate ATP and AMP, thus increasing AMP concentrations from the sub-micromolar range under resting conditions to the lower micromolar range (Frederich and Balschi 2002). To lesser extent, AMP levels also depend on pyrophosphates (cleaving the  $\beta$ -phosphate bond of ATP) and the activity of AMP degradation pathways [AMP-deaminase and 5'-nucleotidase, whose inhibition may be useful to activate AMPK (Kulkarni et al. 2011)]. As a consequence, a decrease in ATP levels by only 10 % translates into a 10- to 100-fold increase in AMP, making AMP an ideal second messenger of energy stress (Fig. 11.13, upper left). Regulation of AMPK activation by the balance between ATP, ADP, and AMP concentrations resembles to what was put forward by Atkinson 40 years ago as “energy charge” regulation (Xiao et al. 2011; Oakhill et al. 2011; Atkinson 1968; Hardie and Hawley 2001).

The molecular basis of allosteric AMPK activation is not yet fully understood, but certainly involves multiple interconnected mechanisms. The nucleotide ratios are sensed at the  $\gamma$ -subunit binding sites (sites 1, 3, and 4), which possess high affinity for AMP and ADP, but less for ATP in its major,  $Mg^{2+}$ -complexed form. AMP or ADP binding to AMPK has several consequences: (1) it makes  $\alpha$ -Thr172 a better substrate for phosphorylation, (2) it protects P- $\alpha$ -Thr172 from dephosphorylation, and (3), only in case of AMP, it exerts direct allosteric activation of AMPK (Xiao et al. 2011; Oakhill et al. 2011; Davies et al. 1995; Suter et al. 2006). All these effects require close communication between the AMP-binding  $\gamma$ - and the catalytic  $\alpha$ -subunit. The three adenylate binding sites participate differentially in these mechanisms. Diverging models have been proposed that involve different structural elements of the  $\alpha$ -subunit (Xiao et al. 2011; Chen et al. 2013a). We and our collaborators have proposed that all these mechanisms involve an AMP- (or ADP)- induced conformational switch within the full-length AMPK complex that is not seen in the X-ray structures of AMPK core complexes solved so far (Chen et al. 2009, 2012; Riek et al. 2008; Zhu et al. 2011).

#### 11.5.4.3 Other Covalent and Non-Covalent Regulations

An increasing number of additional secondary protein modifications adds to the complex scheme of AMPK activation. Myristoylation at Gly2 in the  $\beta$ -subunit increases the sensitivity of AMPK for allosteric activation and promotes Thr172 phosphorylation (Oakhill et al. 2010). Acetylation of  $\alpha$ -subunits is determined by the reciprocal actions of the acetylase p300 and the histone deacetylase 1. AMPK deacetylation promotes its activation via LKB1 interaction (Lin et al. 2012). LKB1 itself is also regulated by acetylation, since deacetylated LKB1 shifts from nucleus to the cytosol, where it forms active complexes with STRAD (Lan et al. 2008). Thus, acetylation is a potentially important factor for activating the LKB1–AMPK pathway (Ruderman et al. 2010), but its role in the heart has not been examined so



far. Glutathionylation at Cys299 and Cys304 in the  $\alpha$ -subunit activates the kinase under oxidative conditions in cellular models and is favored by binding to certain GST isoforms (Klaus et al. 2013; Zmijewski et al. 2010). This latter mechanism may be part of a more general redox regulation of the kinase (Han et al. 2010; Jeon et al. 2012). ROS and RNS activate AMPK, but it is unclear whether this happens via increases in [ADP] and [AMP], or whether noncanonical mechanisms at the level of AMPK (like glutathionylation) or upstream kinases play a role. Vice versa, AMPK regulates NADPH homeostasis and an entire battery of ROS-detoxifying enzymes. Another non-covalent allosteric regulator is glycogen as well as other synthetic branched oligosaccharides that inhibit AMPK activity by binding to the  $\beta$ -GBD domain (McBride et al. 2009).

#### 11.5.4.4 Upstream Regulation in Cardiac (Patho) Physiology

In the heart, AMPK activity is increased by a wide array of signals acting via upstream kinases and modulation of adenylate levels under both pathological and physiological stress and involving various hormones and cytokines (Zaha and Young 2012). Classical physiological stimuli of AMPK are exercise or hypoxia. Both also occur in the heart (Coven et al. 2003; Musi et al. 2005; Frederich et al. 2005) and promote the metabolism of glucose and fatty acids via different downstream targets (see below). However, it is unclear whether this activation is due to altered energy state as in skeletal muscle or rather relies on alternative upstream signaling. AMPK is also involved in the adaptive response of the heart to caloric restriction (Chen et al. 2013b), but nutrient effects in the heart may be more complex (Clark et al. 2004). Possibly, within the physiological range, the role of cardiomyocyte AMPK is different from other cell types, because of the remarkable metabolic stability of this organ maintained by multiple other mechanisms, including the metabolic cycles outlined before.

As pathological stimulus, ischemia is the best studied in form of both no-flow and partial ischemia in isolated perfused animal hearts, as well as regional ischemia due to coronary ligation in vivo (Russell et al. 2004; Kudo et al. 1996; Wang et al. 2009; Paiva et al. 2011; Kim et al. 2011a), for a review, see (Young 2008). They both lead to rapid and lasting AMPK activation. As already mentioned, besides energetic stress, oxidative stress may be a determinant of such activation, acting through different forms of ROS (Sartoretto et al. 2011; Zou et al. 2002). In endothelial cells, it is rather peroxynitrite formation that affects AMPK via the protein kinase C $\zeta$ -LKB1 axis (Zou et al. 2004; Xie et al. 2006b), while in other non-excitable cells it may be rather a ROS-induced Ca<sup>2+</sup> release that triggers the CamKK $\beta$  axis (Mungai et al. 2011). ROS-facilitated glutathionylation of AMPK (see above) as observed in cellular systems represents yet another direct activation mechanism, but still has to be verified in cardiomyocytes (Klaus et al. 2013; Zmijewski et al. 2010). However, the signaling function of ROS may be lost at more intense oxidative stress that simply inactivates AMPK. In models of cardiotoxicity induced by the anticancer drug doxorubicine, AMPK has been

found inactivated in most cases, despite pronounced oxidative, energetic, and genotoxic stress (Tokarska-Schlattner et al. 2005; Gratia et al. 2012). This is probably due to activation of PKB/Akt via DNA-damage signaling kinases that induce the inhibitory cross talk via AMPK $\alpha$ 1-Ser485 phosphorylation. In other situations, also LKB1 may become inactivated (Dolinsky et al. 2009). Stress resulting from many but not all forms of pressure overload also results in AMPK activation, mainly increasing glucose uptake and glycolysis (Tian et al. 2001; Li et al. 2007; Allard et al. 2007; Zhang et al. 2008), as well as changing the gene expression profile (Hu et al. 2011).

Information about the cellular environment and whole-body energy and nutrient state is connected to AMPK signaling via endocrine, paracrine, and autocrine mechanisms. These include a diverse array of hormones and cytokines identified in noncardiac cells that act via largely unknown cellular signaling cascades on AMPK upstream kinases, including adiponectin (Shibata et al. 2004), leptin (Minokoshi et al. 2004), resistin (Kang et al. 2011), ghrelin (Kola et al. 2005), IL6 (Kelly et al. 2004), and CNTF (Watt et al. 2006). Regulation of AMPK by these factors partially depends on the tissue. While in peripheral tissues leptin activates and ghrelin inhibits AMPK in the regulation of fatty acid oxidation and glucose uptake, their effects in hypothalamus are different, since they inhibit (leptin) or stimulate (ghrelin) AMPK-controlled food intake [for reviews see (Kahn et al. 2005; Steinberg and Kemp 2009)]. In the heart, AMPK seems to be involved in the positive effects of adiponectin for cardioprotection during ischemia and for reduced cardiac hypertrophy (Shibata et al. 2004, 2005). For example, AMPK limits accumulation and densification of microtubules that occur in response to hypertrophic stress (Fassett et al. 2013). Also leptin may modulate AMPK in the heart, since impaired leptin signaling correlates with reduced AMPK activation and metabolic defects or reduced postconditioning after ischemia (McGaffin et al. 2009; Bouhidel et al. 2008). Proinflammatory cytokines like IL-6 rather reduce AMPK protein and activation (Ko et al. 2009), although there may be opposite effects in specific tissues like skeletal muscle due to a specific autocrine–paracrine effect (Kelly et al. 2004). Other cytokines with functions in the heart include macrophage migration inhibitory factor (MIF), which is involved in AMPK activation during ischemia and hypoxia and its decrease with age in mice seems to reduce AMPK activation during ischemia (Miller et al. 2008; Ma et al. 2010).

#### **11.5.4.5 Evolution of Cellular Homeostasis Signaling Circuits**

From a phylogenetic perspective, it is interesting to note that AMPK homologues evolved early with eukaryotic life. However, yeast homologues of AMPK lack the direct allosteric AMP-activation, although they already possess the ADP-regulation of the  $\alpha$ -Thr172 phosphorylation state (Mayer et al. 2011). Since such lower eukaryotes neither express a CK/PCr system, it can be concluded that they still tolerate larger fluctuations in energy state. It seems that those more sophisticated regulatory circuits evolved only with multicellular life. It will be interesting to



examine when during metazoan evolution AMP has been established as a second messenger for energy stress and activation of AMPK. Creatine kinase and other closely homologous phosphagen kinases have emerged quite early at the dawn of the radiation of metazoans (Ellington 2001; Ellington and Suzuki 2007). Recently, besides identifying arginine kinase in unicellular organisms, a novel taurocyamine phosphagen kinase has been identified even in a unicellular protist (Uda et al. 2013).

In addition, at least in vertebrates a crosstalk has evolved between AMPK signaling and the Cr/CK system (Neumann et al. 2003; Ju et al. 2012). Although AMPK is not directly activated by Cr as postulated earlier (Ponticos et al. 1998; Ingwall 2002; Taylor et al. 2006), the PCr/Cr ratio will also determine cellular ATP/ADP ratios via the CK reaction and thus indirectly AMPK activation, as well. Knockdown of cytosolic CK activates AMPK (Li et al. 2013), and similar control of AMPK signaling is observed when manipulating the cellular levels of adenylate kinase isoenzymes (Dzeja et al. 2011b). Such indirect mechanisms may also cause the additional AMPK activation observed after Cr supplementation in cellular models of skeletal muscle (Ceddia and Sweeney 2004), in the muscles of patients undergoing exercise programs in different pathological settings (Alves et al. 2012), and in Huntington disease models (Mochel et al. 2012), although these findings need further investigation.

Vice versa, AMPK complexes interact with cytosolic CKs and are able to phosphorylate them (Ponticos et al. 1998; Dieni and Storey 2009). Since this does not affect CK enzyme activity, at least in rodents (Ingwall 2002; Taylor et al. 2006) this phosphophorylation remained enigmatic. Our most recent unpublished data indicate that BB-CK phosphorylation by AMPK may determine subcellular localization of this enzyme which is known to partially associate with ATP-requiring cellular structures and ATPases. In myocytes, active AMPK may also increase cellular Cr uptake by positively acting on Cr transporter (Alves et al. 2012; Darrabie et al. 2011), while an inverse effect was found in kidney epithelial cells (Li et al. 2010). If the latter cells are under energy stress, either physiological or pathological, this mechanism would prevent them to spend additional energy required for Cr uptake from the glomerular filtrate.

### ***11.5.5 Network Connectivity: AMPK Output Signals and Downstream Regulation***

AMPK integrates a large number of signals from inside and outside the cell that carry information on the nutrient and energy state from the cellular to organism level with the aim to mount a coordinated response (Fig. 11.13). This response includes compensation for ATP loss by stimulating catabolic and inhibiting several anabolic pathways, but also control of many other energy-related biological checkpoints in cell growth and proliferation, cell motility and polarity, apoptosis,

autophagy, and central functions like appetite control. To date, about 50 AMPK substrates have been described in different tissues, including metabolic enzymes, transcription (co)factors, and other cellular signaling elements. They all are activated or inactivated by phosphorylation at Thr or Ser residues within a more or less conserved AMPK recognition motif. We will give here only some examples pertinent to heart; more complete descriptions can be found in recent reviews (Hardie et al. 2012a, b; Carling et al. 2012; Steinberg and Kemp 2009).

### 11.5.5.1 Metabolic Pathways

AMPK control of cellular substrate uptake, transport, and metabolism is the historically best described and possibly most important function of AMPK, also in the heart, since it is critical for ATP generation (Fig. 11.5). Activated AMPK stimulates cellular glucose and fatty acid uptake via translocation of GLUT4 (Kurth-Kraczek et al. 1999; Yamaguchi et al. 2005) and FAT/CD36 (van Oort et al. 2009), respectively, to the plasma membrane, involving among others phosphorylation of the Rab-GTPase activating protein TBC1D1 (Frosig et al. 2010). The subsequent substrate flux via glycolysis is increased by phosphorylation and activation of 6-phosphofructosekinase-2 (PFK2), whose product fructose-2,6-bisphosphate is an allosteric activator of the glycolytic enzyme 6-phosphofructokinase-1 (Marsin et al. 2000) and in long term by stimulation of hexokinase II (HKII) transcription (Stoppani et al. 2002). Substrate flux via fatty acid  $\beta$ -oxidation is increased by inhibition of mitochondria-associated acetyl-CoA carboxylase (ACC2), whose product malonyl-CoA is an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme for of mitochondrial fatty acid import and oxidation (Merrill et al. 1997). At the same time, inhibition of cytosolic ACC1 will repress ATP-consuming fatty acid synthesis for which malonyl-CoA is the precursor (Davies et al. 1992). In other organs with multiple anabolic functions like liver, several other anabolic pathways like gluconeogenesis or triglyceride and cholesterol synthesis are inhibited (Bultot et al. 2012; Muoio et al. 1999; Clarke and Hardie 1990).

Active AMPK also affects gene expression of many of these metabolic enzymes by phosphorylation of transcription (co)factors and histone deacetylases (HDACs). Activation of peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1 $\alpha$ ) increases the expression of nuclear-encoded mitochondrial genes that favor mitochondrial biogenesis (Irrcher et al. 2003; Jager et al. 2007), and further catabolic genes including substrate transporters (e.g., GLUT4). Mainly in the liver, expression of several genes in anabolic lipogenesis (e.g., ACC1) and gluconeogenesis is reduced via inhibition of ChREBP or SREBP (Kawaguchi et al. 2002; Li et al. 2011) and CRTC2 or class II HDACs (Koo et al. 2005; Mihaylova et al. 2011), respectively. Cellular redox regulation by AMPK also occurs mainly at the transcriptional level. AMPK directly phosphorylates transcription factor FOXO3, which increases transcription of many genes, mainly in oxidative stress defense (Greer et al. 2007) and activates, possibly more indirectly, class



III deacetylase SIRT1, which deacetylates and thus activates FOXO1/3 and PGC-1 $\alpha$  (Canto et al. 2009).

#### 11.5.5.2 Protein Metabolism, Cell Growth, and Proliferation

AMPK also acts via cross talk with other major cellular signaling hubs. The most important may be the mammalian target of rapamycin complex 1 (mTORC1) which is inhibited by activated AMPK via multiple mechanisms, including phosphorylation of tuberous sclerosis complex protein-2 (TSC2) (Inoki et al. 2003) upstream of mTORC1, or direct phosphorylating the mTORC1 subunit Raptor (Gwinn et al. 2008). This reduces the multiple TORC1 functions in stimulation of protein biosynthesis and cell cycle (Kwiatkowski and Manning 2005) and inhibition of autophagy (Meijer and Codogno 2007). Autophagy is also directly stimulated by AMPK-induced phosphorylation of the protein kinase ULK1 (Kim et al. 2011b; Egan et al. 2011). AMPK further reduces protein synthesis more indirectly by inhibiting eukaryotic elongation factor 2 kinase (eEF2K) (Browne et al. 2004) and downregulating ribosomal RNA (Hoppe et al. 2009) and several cyclins (Wang et al. 2002). Phosphorylation of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup> will both contribute to cell cycle arrest and eventual autophagy (Imamura et al. 2001; Jones et al. 2005; Liang et al. 2007). AMPK also stimulates protein-ubiquitination and proteasome-dependent degradation (Viana et al. 2008; Solaz-Fuster et al. 2008).

#### 11.5.5.3 Cell Contractility, Dynamics, and Shape

AMPK phosphorylates cardiac troponin I (cTnI) during ischemia and thus increases its calcium sensitivity, suggesting that AMPK activation improves myocyte contraction (Oliveira et al. 2012a). Cellular models also suggest that AMPK controls microtubule dynamics through phosphorylation of the microtubule plus end protein CLIP-170 (Nakano et al. 2010) and dynamics of cells and in particular of the mitotic spindle via different indirect mechanisms that increase phosphorylation of the non-muscle myosin regulatory light chain (MRLC) (Lee et al. 2007; Banko et al. 2011).

#### 11.5.5.4 Cellular Ion Homeostasis

The maintenance of ion gradients across cell membranes and intracellular ion homeostasis are further highly energy-demanding processes. Thus it is little surprising that AMPK, like CK, might also regulate these processes. Indeed, different ion transporters are inhibited by AMPK, directly or indirectly, including cystic fibrosis transmembrane conductance regulator Cl-channel (CFTR) (Hallows et al. 2003) and ATP-sensitive potassium (KATP) channel (Kir6.2) (Chang

et al. 2009), and several more cell-type specific ion transporters, like epithelial  $\text{Na}^+$  channel (eNaC) (Carattino et al. 2005), renal  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporter (NKCC2) (Fraser et al. 2007), and neuronal voltage-gated, delayed rectifier  $\text{K}^+$  channel ( $\text{Kv}2.1$ ), whose phosphorylation reduces the frequency of highly energy-consuming action potentials (Ikematsu et al. 2011).

### 11.5.6 Modeling Signaling Networks in Heart and Beyond

Given the complexity and interconnectivity of cell signaling networks, only recently emerging systems biology approaches hold promises for understanding and predicting the higher order properties and behavior of such networks (Arkin and Schaffer (2011) and following papers of this *Cell Leading Edge Review* series). However, the mathematical modeling necessary for such systems approaches is still in its infancy. Modeling needs a solid base of both quantitative and qualitative data, including the spatiotemporal component as outlined above. So far, both bottom-up and top-down systems approaches have been applied to obtain comprehensive databases of protein kinase signaling. Typically, bottom-up, hypothesis-, or model-driven approaches were used to study the role of individual components, also facilitating first studies of dynamic systems properties. More recently, with the broad availability of “omics” approaches, more top-down so-called hypothesis-free studies have mapped interactomes and phosphoproteomes of protein kinases, mainly in yeast (Breitkreutz et al. 2010; Bensimon et al. 2012; Oliveira et al. 2012b). Such large-scale data are necessary to construct first network models needed to advance mathematical modeling in this field. Indeed, progress is also made in the computational methodology and the mathematical description of signaling pathways (Frey et al. 2008; Ideker et al. 2011; Telesco and Radhakrishnan 2012). However, similar modeling approaches with mammalian cells, in particular under physiological and pathological conditions relevant to humans, are still scarce (Benedict et al. 2011; Basak et al. 2012; Rogne and Tasken 2013), except a strong history of modeling in cardiac electrophysiology (Amanfu and Saucerman 2011). Such models would be highly valuable for in silico drug target identification, drug screening, and development (Benedict et al. 2011). Important steps in such approaches are (1) to establish a network structure, (2) to obtain quantitative datasets for basic systems properties, (3) to generate dynamic mathematical models, and (4) to test and iteratively improve the models by prediction and experimental verification of systems perturbations (Frey et al. 2008). First models also including AMPK signaling are currently emerging (Marcus 2008; Sonntag et al. 2012), but sustained interdisciplinary efforts in the field will be necessary to obtain models that allow meaningful predictions of AMPK systems behavior.

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