Coupling of cell energetics with membrane metabolic sensing: Integrative signaling through creatine kinase phosphotransfer disrupted by M-CK gene knockout

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Transduction of metabolic signals is essential in preserving cellular homeostasis. Yet, principles governing integration and synchronization of membrane metabolic sensors with cell metabolism remain elusive. Here, analysis of cellular nucleotide fluxes and nucleotide-dependent gating of the ATP-sensitive K\(^{+}\) (K\(_{\text{ATP}}\)) channel, a prototypic metabolic sensor, revealed a diffusional barrier within the submembrane space preventing direct reception of cytosolic signals. Creatine kinase phosphotransfer, captured by \(^{18}\text{O}\)-assisted \(^{31}\text{P}\) NMR, coordinated tightly with ATP turnover, reflecting the cellular energetic status. The dynamics of high-energy phosphoryl transfer through the creatine kinase relay permitted a high-fidelity transmission of energetic signals into the submembrane compartment synchronizing K\(_{\text{ATP}}\) channel activity with cell metabolism. Knockout of the creatine kinase \(M-C\) gene disrupted signal delivery to K\(_{\text{ATP}}\) channels, and generated a cellular phenotype with increased electrical vulnerability. Thus, in the compartmentalized cell environment, phosphotransfer systems shunt diffusional barriers and secure regimented signal transduction integrating metabolic sensors with the cellular energetic network.
Maintenance of cellular homeostasis critically depends on the ability of the cell to adjust diverse energy-dependent processes in response to metabolic challenge (1-3). This requires efficient monitoring of cellular metabolism, secure delivery of information to energetic sensors and accurate translation of metabolic signals into cellular response (4-10). Advances have been made in resolving the molecular identity and regulatory properties of energetic signal transducers (11, 12), yet mechanisms that integrate and synchronize metabolic sensors with cell metabolism are only partially understood (13-15).

ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels are membrane metabolic sensors, which act as alarm systems to adjust cell electrical activity and regulate vital functions as diverse as hormone secretion, neurotransmitter release or cytoprotection (6, 9, 11, 16-20). K\(_{\text{ATP}}\) channels are expressed in high density in metabolically active tissues, in particular heart muscle, where the pore-forming Kir6.2 protein assembles with the regulatory sulfonylurea receptor SUR2A subunit to form functional hetero-octameric complexes (21-23). While ATP closes K\(_{\text{ATP}}\) channels by interacting with Kir6.2, metabolic sensing seems to proceed through interactions of ATP/ADP with nucleotide-binding domains of SUR (9, 16, 24-27). In this regard, active membrane ATPases constantly reduce the local ATP concentration setting the submembrane ATP/ADP ratio distinct from that of the “bulk” cytosol (15, 28-31). However, such independent nucleotide fluctuations within a particular cell compartment (15, 31) would hamper proper recognition of cellular signals rendering K\(_{\text{ATP}}\) channels ineffective metabolic sensors.

In response to metabolic alterations, the membrane content of polyphosphoinositides has been implicated in defining the ATP-sensitivity of cardiac K\(_{\text{ATP}}\) channels (32). Yet, altered K\(_{\text{ATP}}\) channel sensitivity \textit{per se} may not provide an efficient mechanism of metabolic signal transduction as drastic reduction in the channel responsiveness to ATP, induced by mutation of Kir6.2, has no apparent consequences on channel behavior and/or membrane electrical activity in metabolically competent cardiac cells (33). Rather, coordination of membrane sensor function with the cellular metabolic status mandates effective transfer of energetic signals between intracellular compartments (2, 15). Cells with high and fluctuating energy demands, such as cardiomyocytes, possess catalyzed phosphotransfer circuits that facilitate energetic signaling between sites of ATP production and utilization (3, 5). Emerging evidence suggests that phosphotransfer networks can process metabolic information for
delivery to metabolic sensors, thereby serving a critical role in cellular homeostasis (2, 3, 34, 35). In this way, the phosphotransfer enzyme adenylate kinase physically associates with $K_{ATP}$ channel proteins to facilitate communication of mitochondrial signals and promote channel opening in stress (34). A related signal delivery function has been suggested for the most active phosphotransfer enzyme in the myocardium, creatine kinase, which could control $K_{ATP}$ channel closure and prevent accidental channel opening (3, 36, 37). Isoforms of creatine kinase are found in distinct intracellular compartments, including membranes where $K_{ATP}$ channels reside (13, 38). Substrates of creatine kinase regulate nucleotide-dependent $K_{ATP}$ channel gating, and can overcome potassium channel opener-induced channel activation (36, 37, 39). In fact, in opener-primed cardiomyocytes inhibition of creatine kinase reduces the effect of mitochondrial uncoupling on $K_{ATP}$ channel activity (40). Although an intimate relationship between phosphotransfer enzymes and the channel itself has been suggested (3, 36, 37, 40, 41), the requirement for creatine kinase phosphotransfer in synchronizing metabolic sensor function in response to fluctuations in the cellular metabolic state has not been defined.

Here, we demonstrate that the dynamics of high-energy phosphoryl transfer through the creatine kinase system coordinates $K_{ATP}$ channel activity with cellular metabolism contributing to an integrative mechanism for delivery of energetic signals to the membrane sensor. Deletion of the $M-CK$ gene, which encodes the major creatine kinase isoform, disrupted creatine kinase-dependent signal delivery to $K_{ATP}$ channels and generated a phenotype with increased electrical vulnerability.

**EXPERIMENTAL PROCEDURES**

*Creatine kinase knockout* - Mice lacking the M-creatine kinase (M-CK) isoform were derived from embryonic stem cells carrying a replacement mutation in the $M-CK$ gene (1). Inactivation of M-CK expression was achieved by homologous DNA recombination with a HygroB cassette vector used to replace exon 2 and parts of introns 1 and 2 in the $M-CK$ gene. Homozygous M-CK-knockout mice were compared with age-matched wild-type controls.

*Channel recording* - Cardiomyocytes, isolated from wild-type and M-CK knockout mice or guinea-pig ventricles (34), were bathed in (mM) KCl 140, MgCl$_2$ 1, EGTA 5, HEPES-KOH 5 (pH 7.3).
Patch electrodes (7-10 MΩ) were filled with (mM) KCl 140, CaCl₂ 1, MgCl₂ 1, HEPES-KOH 5 (pH 7.3). For the open cell-attached patch, bath solution was supplemented with glucose (1 g/l), malic acid (5 mM) and pyruvic acid (5 mM). Following seal formation with the patch pipette, cell permeabilization was achieved by digitonin (5 - 8 µg/ml) applied through a second pipette (filled with 5 µg/ml propidium iodide and 0.5 µg/ml rhodamine). Under ultraviolet light, rhodamine served for visualization of solution flow, and propidium iodide staining of the cell nucleus indicated formation of the open cell-attached patch configuration (36, 37). Channel activity was measured at -60 mV.

*Phosphotransfer scanned by NMR spectroscopy* - ¹⁸O-assisted ³¹P NMR is based on incorporation of ¹⁸O, provided from ¹⁸O-water, into cellular phosphates proportionally to the rate of enzymatic reactions involved (42, 43):

\[
\begin{align*}
\text{³¹P \text{H}_{2}\text{O}} & \quad \text{⁰ATP} \rightarrow [\text{¹⁸O}] \text{P}_i + \text{ADP} & \text{ATP HYDROLYSIS} \\
[\text{¹⁸O}] \text{P}_i + \text{ADP} & \rightarrow [\text{¹⁸O}] \text{γATP} & \text{ATP SYNTHESIS} \\
[\text{¹⁸O}] \text{γATP} \text{Cr} & \rightarrow [\text{¹⁸O}] \text{CrP} + \text{ADP} & \text{CREATINE KINASE PHOSPHOTRANSFER}
\end{align*}
\]

The ¹⁸O-phosphoryl labeling procedure detects only newly generated molecules containing ¹⁸O-labeled phosphoryls reflecting net flux through an individual phosphotransfer pathway. Hearts were perfused at 37°C with 95% O₂/5% CO₂ saturated buffer (in mM: 123 NaCl, 6 KCl, 2.5 CaCl₂, 0.5 EDTA, 19 NaHCO₃, 1.2 MgSO₄, 11 glucose and 20 U/l insulin). Hypoxia was induced with 95%N₂/5% CO₂ gassed buffer, to reduce partial oxygen pressure to 20-30 mmHg. ¹⁸O labeling was achieved using the buffer supplemented with 40% of ¹⁸O-H₂O (Isotec) for 30 s. During this time ¹⁸O labeling is still within the initial pseudolinear phase of the labeling kinetic curve, which reaches full saturation only after 2 min following application of ¹⁸O-H₂O. Hearts freeze-clamped and extracted in 600 mM HClO₄ and 1 mM EDTA. ¹⁸O-induced shifts in ³¹P NMR spectra of ATP and creatine phosphate were recorded at 242.9 MHz in a Bruker 14 T spectrometer (42, 43), and phosphotransfer fluxes calculated as described (38).

*Enzymatic activity* - Hearts homogenized in (in mM) 10 HEPES, 1 EGTA, 1 DTT, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride and 1 µg/ml leupeptin (pH 7.4) were spun at 5,000 g. Supernatant was centrifuged at 100,000 g, and membrane pellets suspended by sonication in (in mM) 20 HEPES
(pH 7.4), 140 NaCl, 5 KCl, 2 MgCl₂, 0.5 dithiothreitol, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride and 2 µg/ml leupeptin. Creatine kinase activity was determined with a coupled enzyme assay (38).

Action potentials - Mouse hearts were perfused at 90 mmHg with (in mM) NaCl 108, KCl 5, HEPES 5, glucose or deoxyglucose 5, sodium acetate 20, MgCl₂ 1, CaCl₂ 2, malate 1, pyruvate 5 and insulin 5 U/l (pH 7.4, 37°C). Monophasic action potentials were recorded from the left ventricular epicardial surface using a probe (EP Technologies) while pacing at 130 ms cycle length and 10 ms pulse width (Accupulser, WPI). In guinea-pig hearts, action potentials were measured without pacing.

Allosteric model of channel gating – Nucleotide-dependent K<sub>ATP</sub> channel gating was simulated by an allosteric model where four identical binding sites for ATP and ADP co-exist within the octameric stoichiometry of the K<sub>ATP</sub> channel complex (16, 22). Binding of ATP to the pore-forming Kir6.2 subunit inhibits channel opening (24, 25), whereas binding of ADP to the regulatory SUR subunit antagonizes ATP-binding to Kir6.2 (6, 26, 44). Distribution of channel species (D<sub>i</sub>; i=0 to 4) with 0-4 ADP bound molecules was:

\[
\frac{D_1}{D_0} = \frac{4\cdot[ADP]}{k_{ADP}}, \quad D_2 = \frac{3\cdot[ADP]}{2k_{ADP}}, \quad D_3 = \frac{2\cdot[ADP]}{3k_{ADP}}, \quad D_4 = \frac{[ADP]}{4k_{ADP}}, \quad \sum_{j=1}^{4} D_j = 1
\]  

(1)

with the percentage of D<sub>i</sub> species expressed as a function of ADP concentration:

\[
D_i = \frac{4\cdot D_0 [ADP]}{k_{ADP}}; \quad D_2 = 6\cdot D_0 \left(\frac{[ADP]}{k_{ADP}}\right)^2; \quad D_3 = 4\cdot D_0 \left(\frac{[ADP]}{k_{ADP}}\right)^3; \quad D_4 = D_0 \left(\frac{[ADP]}{k_{ADP}}\right)^4, \quad \text{where}
\]

(2)

\[
D_0 = \left[1 + 4\cdot \frac{[ADP]}{k_{ADP}} + 6\cdot \left(\frac{[ADP]}{k_{ADP}}\right)^2 + 4\cdot \left(\frac{[ADP]}{k_{ADP}}\right)^3 + \left(\frac{[ADP]}{k_{ADP}}\right)^4\right]^{-1}
\]

and k<sub>ADP</sub> the dissociation constant of ADP from SUR, independent from ATP binding. Analogously to Equations 1 and 2, the distribution of channel species (T<sub>i</sub>) with 0 to 4 ATP bound molecules was derived as \(\sum_{i=0}^{4} T_i = D_j, j = 0 - 4\), with \(k_0\) and \(k_1\) representing dissociation constants for ATP binding to Kir6.2 in the absence and presence of ADP at the associated SUR (Fig. 1A-B). The best fits of experimental data from ATP-induced K<sub>ATP</sub> channel inhibition in the absence of ADP, at saturating ADP and at below saturating ADP revealed respectively the values for \(k_0, k_1\) and \(k_{ADP}\), with more than one ATP required to close the channel octamer.
Computation of diffusional restriction between cell compartments - Diffusional restriction was estimated by integrating membrane ATPase activities and diffusional nucleotide fluxes (Fig. 1C). Membrane ATP consumption ($J_{\text{ATPase}}$) was simulated as a Michaelis-Menten reaction with the Michaelis constant at 0.05 mM (45). Sarcolemmal ATPase activity (1800 nmol/min/g w wt) was derived from total ATPase activity in working hearts measured by $^{18}$O-assisted $^{31}$P NMR (300 nmol/min/mg protein), assuming that 120 mg of protein (with 1 mg of sarcolemmal protein) is contained in 1 g of tissue and that ~5% of total energy is consumed by sarcolemmal ATPases (45). Nucleotide diffusion (with a coefficient $D$) was calculated according to Fick’s law as one-dimensional flux (through total cell area in 1 g of tissue, $S$) perpendicular to the membrane. Diffusional flux for ATP was:

$$J^{\text{diff}}_{\text{ATP}}(x) = -DS \frac{\partial [\text{ATP}(x)]}{\partial x}$$

where $J_{\text{ATP}}(x)$ is ATP flux at distance $x$. At steady-state, with $J_{\text{ATP}}$ constant, $C = -DS \Delta x$, defining:

$$J^{\text{diff}}_{\text{ATP}} = C([\text{ATP}]_b - [\text{ATP}]_{\text{sub}})$$

where $[\text{ATP}]_b$ and $[\text{ATP}]_{\text{sub}}$ are cytosolic “bulk” and subsarcolemmal ATP concentration, respectively. Diffusional flux for ADP was described analogously as in Equation 4. $[\text{ATP}]_{\text{sub}}$ and $[\text{ADP}]_{\text{sub}}$, as a function of $[\text{ATP}]_b$ (Fig. 1D), were defined from:

$$C([\text{ATP}]_b - [\text{ATP}]_{\text{sub}}) - J_{\text{ATPase}} = 0$$
$$C([\text{ADP}]_b - [\text{ADP}]_{\text{sub}}) + J_{\text{ATPase}} = 0$$

RESULTS

Metabolic sensing in the submembrane compartment - The defining property of cardiac $K_{\text{ATP}}$ channels as membrane metabolic sensors is their overt inhibition by ATP, which can be antagonized by MgADP (Fig. 1A). $K_{\text{ATP}}$ channels adopt their highest sensitivity to ATP in the absence of MgADP, and convert to a range of lower ATP-sensitivities with increasing concentrations of MgADP (Fig. 1A). The regulatory SUR2A subunit harbors an intrinsic ATPase activity (36, 44) that facilitates conformational
transitions imparting low or high ATP-sensitivity to the \( \text{K}_{\text{ATP}} \) channel complex (37). MgADP prolongs the lifetime of the conformation associated with reduced sensitivity to ATP (37). Allosteric modeling, which integrated \( \text{K}_{\text{ATP}} \) channel stoichiometry and channel-nucleotide interactions (Fig. 1B), demonstrated that on saturating ADP-binding sites (at >100 \( \mu \text{M} \) ADP) no further reduction in ATP sensitivity can be achieved (Fig. 1A-B), in accord with the efficacy of ADP to antagonize ATP-induced channel inhibition (47). For MgADP to open at least 1% of \( \text{K}_{\text{ATP}} \) channels, required for significant action potential shortening at 6-10 mM of cytosolic ATP (47-50), ATP at the channel site needs to be reduced to <3 mM (Fig. 1C-D). Local drop in ATP could be generated by membrane ATPases (30, 51), including ATP hydrolysis by the \( \text{K}_{\text{ATP}} \) channel itself (36, 37, 44), provided that nucleotide mobility between the cytosol and submembrane is limited (31; Fig. 1C). Calculations, based on membrane ATPase activity and nucleotide gradients between the cytosolic “bulk” and subsarcolemmal space, revealed a strong diffusional hindrance with an apparent diffusion coefficient \( D=2.3\cdot 10^{-11} \text{cm}^2/\text{s} \). This value, five orders of magnitude lower than values for nucleotide diffusion in the cytosol (52), is in line with the restricted diffusion of molecules previously observed in the structurally crowded submembrane space of living cells (51, 53). Such a diffusional barrier implies virtual confinement of the metabolic sensor within the submembrane zone, impeding direct exposure to cytosolic signals. Thus, integration of \( \text{K}_{\text{ATP}} \) channel activity with cell metabolism requires efficient mechanisms able to shunt diffusional restrictions for proper delivery of energetic signals.

Creatine kinase phosphotransfer synchronized with cellular energy turnover sets nucleotide-dependent \( \text{K}_{\text{ATP}} \) channel gating - Creatine kinase molecules are spatially arranged between cellular sites of ATP-production and utilization, providing an integrated network for high-energy phosphoryl conduction (1, 3, 38, 54-56). Here, net phosphotransfer flux through the creatine kinase system was captured in intact heart using \(^{18}\text{O} \)-assisted \(^{31}\text{P} \) NMR, and was found to be tightly synchronized with cellular ATP turnover (Fig. 2A). Labeling of phosphoryl oxygens in creatine phosphate, which reflects

\[ D=C\cdot \Delta x/S \] was derived from \( C=0.45 \text{ cm}^3/\text{min/g w wt} \) (Equation 5, Fig. 1D), assuming a subsarcolemmal space width (\( \Delta x \)) of \( 10^{-5} \text{ cm} \), and a total surface of cells in 1 g of tissue (\( 10^{12} \text{ \mu m}^3 \)) \( S=3200 \text{ cm}^2/\text{g w wt} \) for an average cardiac cell (\( 10\times 20\times 100 \text{ \mu m} \)) with a volume of \( 20000 \text{ \mu m}^3 \) and a surface of \( 6400 \text{ \mu m}^2 \).
net flux through creatine kinase, paralleled that of \( \gamma \)-phosphate in ATP, an indicator of total cellular high-energy flux (Fig. 2A). On average, the percentage of \(^{18}\)O in creatine phosphate was comparable to that of \( \gamma \)-ATP, i.e., 55\(\pm\)4\% (n=5) versus 61\(\pm\)3\% (n=5), respectively (Fig. 2B). Such a vigorous creatine kinase catalyzed phosphotransfer would rapidly dissipate local nucleotide gradients, and could therefore transmit cellular metabolic signals to membrane metabolic sensors. In fact, creatine kinase catalysis was found high in the cardiac membrane fraction, i.e., 6.2\(\pm\)0.6 \(\mu\)mol/min/mg (n=3), and was sensitive to the conventional creatine kinase inhibitor 2-4-dinitrofluorobenzene (DNFB) which reduced such activity to 0.5\(\pm\)0.1 \(\mu\)mol/min/mg (n=3). To assess the role of creatine kinase flux in regulating \( K_{\text{ATP}} \) channels as metabolic sensors, channel behavior was measured in the absence and presence of creatine kinase phosphotransfer. To maintain a relative integrity of the cellular infrastructure, cardiomyocytes were permeabilized by local and brief application of digitonin to the region of the cell distal from the patched area (Fig. 2C). In such open cell-attached patch configuration, removal of creatine phosphate inactivated creatine kinase phosphotransfer and induced an aberrant sensitivity of \( K_{\text{ATP}} \) channels towards ATP (Fig. 2D). Indeed, ATP at 100 \( \mu\)M failed to inhibit \( K_{\text{ATP}} \) channels (Fig. 2D), a concentration that keeps channels closed in excised membrane patches (Fig. 2E). The reduced ATP-sensitivity indicates that, despite clamped “bulk” nucleotide concentrations by continuous cell perfusion, local levels of ATP are decreased and ADP increased in an environment of active membrane ATPases. Activation of creatine kinase phosphotransfer, by addition of creatine phosphate, restored the \( K_{\text{ATP}} \) channel responsiveness to ATP (Fig. 2D) presumably through scavenging ADP and dissipating the membrane ATPase-induced nucleotide gradient. Creatine phosphate had no significant effect on its own, but secured \( K_{\text{ATP}} \) channel closure in open cell-attached patches in the presence of low ATP concentrations which in inside-out patches produced only partial channel inhibition (Fig. 2E and 2F). In the presence of creatine kinase substrates, inhibition of creatine kinase phosphotransfer by DNFB in permeabilized cells uncoupled \( K_{\text{ATP}} \) channels from phosphotransfer regulation (Fig. 2F). Coupling was restored by providing purified creatine kinase to bypass the irreversible inhibition of endogenous creatine kinase by DNFB (Fig. 2F). Thus, the creatine phosphate/creatine kinase system is a determinant of the \( K_{\text{ATP}} \) channel sensitivity to ATP. In permeabilized cells, in the absence of creatine
kinase flux ($J_{CK} = 0$), the IC$_{50}$ for ATP-induced inhibition was 270±2 μM (n=4; Fig. 2E), close to the value measured in the presence of saturating MgADP in excised patches (Fig. 1A). Active creatine kinase flux ($J_{CK} >> 0$) significantly reduced the IC$_{50}$ to 7±1 μM (n=4), even below the sensitivity of the channel towards ATP seen in excised patches (Fig. 2E). Thus, creatine kinase flux can shunt nucleotide gradients between the “bulk” and subsarcolemmal space, and facilitate delivery of metabolic signals that translate into K$_{ATP}$ channel-dependent sensing of the cellular energetic state.

Creatine kinase phosphotransfer dynamics transduce metabolic stress-induced signals into K$_{ATP}$ channel-driven membrane electrical events - In normoxia, when cardiac K$_{ATP}$ channels are closed, vigorous incorporation of $^{18}$O atoms into creatine phosphate reflected the high creatine kinase phosphotransfer rate of the myocardium (Fig. 3A). Hypoxia markedly reduced creatine kinase flux, from 279±8 nmol CrP/min/mg protein (n=3) to 64±17 nmol CrP/min/mg protein (n=3; p<0.05), indicating a ~75% decrease in creatine kinase phosphotransfer (Fig. 3A). This reduction in creatine kinase phosphotransfer reflects a four-fold drop, from 36±1 to 9±2 nmol/mg protein, in creatine phosphate levels following hypoxia. Thus, under hypoxic stress, creatine kinase has a reduced ability to equilibrate nucleotide levels between the cytosol and subsarcolemma. Estimated subsarcolemmal concentrations of ATP ([ATP]$_{sub}$) and ADP ([ADP]$_{sub}$) were deduced by integrating diffusional restriction (C), “bulk” concentrations ([ATP]$_b$, [ADP]$_b$), and membrane ATPase activity ($J_{ATPase}$; see equation 5) with creatine kinase flux ($J_{CK}$):

$$\frac{C([ATP]_b - [ATP]_{sub}) - J_{ATPase} + J_{CK}}{C([ADP]_b - [ADP]_{sub}) + J_{ATPase} - J_{CK}} = 0$$

In normoxia, with creatine kinase flux compensating for membrane ATPase activity ($J_{ATPase}=J_{CK}$), nucleotide gradients were dissipated with [ATP]$_b$=[ATP]$_{sub}$ and [ADP]$_b$=[ADP]$_{sub}$ (Fig. 3B). With equilibrated [ATP]$_b$ and [ATP]$_{sub}$, at 6-10 mM, K$_{ATP}$ channels remained closed as the concentration-response curve for ATP-induced channel inhibition is far below the actual intracellular ATP levels (Fig 3B). In hypoxia, reduced creatine kinase flux ($J_{ATPase} >> J_{CK}$) unmasked the membrane ATPase-induced drop in ATP ([ATP]$_b$>[ATP]$_{sub}$) and increase in ADP ([ADP]$_b$<[ADP]$_{sub}$) in the subsarcolemmal space (Fig. 3C). In this way, metabolic challenge resulting in altered creatine kinase phosphotransfer could
bring subsarcolemmal nucleotide levels to a range that now lies in the steeper portion defining ATP-dependent channel gating, securing effective signal delivery to $K_{\text{ATP}}$ channels (Fig. 3C). A drop of 75% in creatine kinase phosphotransfer ($\Psi_{\text{CK}}$), observed in hypoxia (Fig. 3A) at cytosolic ATP<6 mM, would translate into activation of >1% of $K_{\text{ATP}}$ channels sufficient for significant action potential shortening in a cardiac cell (50), as computed from the allosteric model of nucleotide-dependent channel gating (Fig. 1B) taking into account nucleotide diffusion, membrane ATPase and creatine kinase fluxes (Fig. 3D). Indeed, cardiac action potential duration was significantly decreased from 230±4 ms prior, to 130±7 ms following hypoxic stress (Fig. 3E). Thus, the dynamics of creatine kinase phosphotransfer, governed by the cellular metabolic condition, determine the percentage of open $K_{\text{ATP}}$ channels and provide a mediator translating cellular energetic signals into membrane electrical events.

**Knockout of creatine kinase disrupts signal delivery to the metabolic sensor** - Cytosolic creatine kinase (M-CK) is the major creatine kinase isoform in the heart (1). Deletion of the M-CK gene blunted creatine kinase phosphotransfer, and essentially eliminated creatine kinase activity in the sarcolemma (Fig. 4A). In wild-type cardiomyocytes, $K_{\text{ATP}}$ channel activity was highly sensitive to creatine kinase-mediated channel inhibition, revealed from titration with creatine phosphate ($IC_{50}$=94±5 $\mu$M, n=5; Fig. 4B-C). This indicates tight integration of creatine kinase-catalyzed energetic signaling with $K_{\text{ATP}}$ channel activity. In contrast, in cells lacking M-CK, $K_{\text{ATP}}$ channels were uncoupled from the cellular energetic infrastructure, and were no longer sensitive to creatine phosphate regulation (Fig. 4B-D). Thus, cells lacking the creatine kinase phosphotransfer system display a defective regulation of nucleotide-gated membrane functions.

**Fidelity in membrane metabolic sensing lost with deletion of the M-CK gene** - Besides creatine kinase, additional phosphotransfer pathways, such as the glycolytic system, have been identified as interrelated components of the cellular energetic network (2, 3, 5). Here, in wild-type cardiomyocytes, inhibition of phosphoryl delivery through the glycolytic system by deoxyglucose did not elicit a $K_{\text{ATP}}$ channel response (Fig. 5A), suggesting privileged control of this metabolic sensor by creatine kinase. In contrast, in cells lacking M-CK, metabolic stress induced with deoxyglucose triggered $K_{\text{ATP}}$ channel opening (Fig. 5B-C). In fact, aberrant coupling of $K_{\text{ATP}}$ channels with cellular metabolism in creatine
kinase-knockout hearts generated a phenotype with electrical instability manifested by premature shortening of action potentials in response to deoxyglucose (Fig. 5D-E). The rate of stress-induced action potential shortening was significantly faster in creatine kinase-knockout (0.042±0.005 min⁻¹, n=4) than wildtype (0.016±0.004 min⁻¹, n=4) hearts (p<0.05; Fig. 5D). Accordingly, action potential duration, measured at 90% of repolarization, was essentially unchanged (94±4 % of control value, n=4) in wildtype, but was reduced, by 39±9 % (n=4), in the M-CK knockout following a 7-min long application of deoxyglucose (Fig. 5E). Thus, creatine kinase phosphotransfer is required for proper linkage of cellular energetics with membrane excitability.

DISCUSSION

Metabolic signal transduction governs vital functions that enable cells to respond to metabolic challenges. But how the operation of metabolic sensors is orchestrated to accurately sense the cellular energetic status has remained a long-standing enigma. Using cardiac K_ATP channels as prototypic membrane metabolic sensors, we demonstrate that phosphotransfer enzyme-catalyzed metabolic signal delivery synchronizes channel gating with cell energetics. Genetic disruption of the creatine kinase pathway generated a phenotype with increased electrical vulnerability, underscoring the significance of an intact intracellular phosphotransfer network in integrating metabolic signaling.

The extremely low diffusional flux of nucleotides, estimated here in the subsarcolemmal space, indicates that K_ATP channels are virtually secluded from cellular “bulk” nucleotide oscillations. Restricted metabolite mobility in the premembrane area could be due to molecular crowding and reduction in the free diffusional space as previously suggested for different cellular compartments (13-15, 53). In fact due to the “fuzzy space” in the submembrane (31), channel gating would be relegated to local fluctuations of nucleotides (“metabolic background noise”), independent of the cellular metabolic status. Instead of random fluctuations in adenine nucleotides, which would distort energetic signaling, we provide direct evidence that the creatine kinase phosphotransfer system controls exchange of nucleotides securing signal processing between the subsarcolemmal space and cytosolic compartment.
Indeed, the dynamics of creatine kinase flux closely followed total ATP turnover, indicating tight coupling between creatine kinase phosphotransfer and the cellular metabolic state (38, 43). Present throughout the cell, creatine kinase reactions form a phosphotransfer relay able to respond to changes in the cellular metabolic state and propagate metabolic waves between cellular compartments (3, 57, 58). Such catalyzed phosphotransfer can deliver metabolic signals at a rate exceeding simple diffusion (38, 58). Accordingly, here, under the normal status of cell metabolism, vigorous creatine kinase phosphotransfer dissipated local nucleotide gradients created by membrane ATPases and diffusional restrictions in the channel environment, keeping $K_{\text{ATP}}$ channels predominantly closed. Under metabolic stress, however, reduced creatine kinase phosphotransfer unmasked nucleotide changes in the channel vicinity alerting $K_{\text{ATP}}$ channels to adjust membrane excitability. Reduced creatine kinase flux under metabolic insult is known to be associated with concomitant up-regulation of adenylate kinase phosphotransfer, which catalyzes the conversion of ATP to ADP at the channel site (34, 57). Such interplay between phosphotransfer pathways effectively amplifies the metabolic signal translating into $K_{\text{ATP}}$ channel opening and ultimately shortening of the cardiac action potential under stress (19, 59). Thus, membrane metabolic sensors respond to the dynamics of cellular phosphotransfer flux, reflecting with high fidelity the energetic status of a cell.

Integration of phosphotransfer with $K_{\text{ATP}}$ channels appears critical in supporting metabolic signaling. Knockout of the dominant creatine kinase isoform, M-CK, disrupted $K_{\text{ATP}}$ channel regulation by creatine phosphate. This produced a cellular phenotype characterized by increased electrical instability, in line with observations that muscles lacking creatine kinase genes display abnormal contractile response and reduced energetic efficiency (1, 4, 54, 55). Moreover, coupling of creatine kinase with $\text{Ca}^{2+}$-ATPases of the sarcoplasmic reticulum, which is essential in securing $\text{Ca}^{2+}$ handling and proper kinetics of intracellular $\text{Ca}^{2+}$ signals, is compromised following deletion of creatine kinase genes (60). In this regard, creatine kinase can also functionally couple with $K_{\text{ATP}}$ channels through direct creatine kinase-dependent regulation of the ATPase catalytic cycle harbored within SUR, the channel regulatory subunit (36, 37). The ATP hydrolysis cycle at SUR drives conformational transitions associated with distinct outcomes on channel behavior, with creatine kinase promoting disengagement
of the MgADP-bound state and $K_{\text{ATP}}$ channel closure (37). Thus, nucleotide exchange between cellular phosphotransfer catalyzed by creatine kinase and membrane ATPases, including the channel’s own ATPase, provides a mechanistic basis for coupling cell energetics with metabolic signal transduction.

Along with creatine kinase, distinct phosphotransfer systems can also efficiently communicate energetic signals to metabolic signal transducers, and regulate ATP-sensitive cellular components (3, 34, 61). The interrelationship between intracellular energetic pathways is revealed upon deletion of the $M-C K$ gene, which translated into redistribution of metabolic flux through glycolytic enzymes (56, 62). In accord with the adaptive potential of phosphotransfer pathways (1, 5, 42), such energetic remodeling was sufficient to maintain apparently normal $K_{\text{ATP}}$ channel gating in the absence of metabolic challenge in the M-CK knockout heart. However, knockout of M-CK did produce increased electrical vulnerability manifested by premature action potential shortening in hearts stressed by inhibition of glycolytic enzymes. Thus, an intact phosphotransfer network is a prerequisite for optimal decoding of energetic signals securing adequate function of a metabolic sensor. The significance of these findings is underscored in human disease where compromised creatine kinase phosphotransfer has been associated with cardiac electrical instability (63), and extrapyramidal movement disorders (64).

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Figure legends

**FIG. 1.** Nucleotide-dependent gating of $K_{\text{ATP}}$ channels. (A-B) MgADP antagonizes ATP-induced inhibition of cardiac $K_{\text{ATP}}$ channels (*inset*). In excised patches, the ATP sensitivity of $K_{\text{ATP}}$ channels was defined by an IC$_{50}$ of 27±5 µM in the absence (open triangles) versus 270±19 µM in the presence (closed circles) of 100 µM ADP (n=3-9). Relative channel activity (*curves* in A) constructed based on an allosteric model of nucleotide-dependent $K_{\text{ATP}}$ channel gating (*B*), and expressed as a probability for the channel to be in an open state (columns $T_0$-$T_1$). In the absence of ADP, channels adopt the highest sensitivity to ATP (row $D_0$) defined solely by the microscopic dissociation constant $k_0$=45 µM (*curve* 1 in A). At saturating ADP concentrations, $K_{\text{ATP}}$ channels convert to channel species with the lowest ATP sensitivity (row $D_4$) defined solely by $k_1$=450 µM (*curve* 4 in A). $k_{\text{ADP}}$ (12.5 µM) was determined at different concentrations of ADP (*curve* 1: 0; 2: 10; 3: 50; 4: 100; 5: 500; 6: 1000 µM ADP). (C) Membrane ATPases and diffusional restrictions generate nucleotide gradients between cytosol and subsarcolemma, generating lower ATP and higher MgADP at the channel site. (D) Relationships between “bulk” and subsarcolemmal adenine nucleotide levels when nucleotide diffusional fluxes are at steady state ($J_{\text{ATP}}^{\text{diff}}=-J_{\text{ADP}}^{\text{diff}}$). Subsarcolemmal ATP (ATP$_{\text{sub}}$) and ADP (ADP$_{\text{sub}}$) were calculated from Equation 5 (see Experimental Procedures). At “bulk” ATP (ATP$_b$) between 4-10 mM, ATP$_{\text{sub}}$ follows ATP$_b$, while ADP$_{\text{sub}}$ remains constant due to saturation of ATPase activity. At ATP$_b$=7 mM, ATP$_{\text{sub}}$=3 mM, sufficient to activate 1% of $K_{\text{ATP}}$ channels at saturating ADP$_b$ (*inset*, curves from A expressed as % of open $K_{\text{ATP}}$ channels).

**FIG. 2.** Creatine kinase phosphotransfer determines nucleotide-dependent $K_{\text{ATP}}$ channel gating. (A) Spectra of $^{18}$O-labeled γATP and creatine phosphate (CrP) captured by $^{18}$O-assisted $^{31}$P NMR. Incorporation of $^{18}$O induces an isotope shift in the $^{31}$P spectra of γATP and CrP. $^{16}$O, $^{18}$O$_1$, $^{18}$O$_2$, $^{18}$O$_3$ designate γATP and CrP phosphoryls containing 0-3 atoms of $^{18}$O. γATP is recorded as a doublet due to homonuclear scalar coupling between β and γ phosphates. $^{18}$O labeling of CrP and γATP are comparable in magnitude, indicating that creatine kinase is responsible for transfer of the majority of newly synthesized ATP. (B) Average incorporation of $^{18}$O into phosphoryls of CrP reflects creatine
kinase-catalyzed phosphotransfer, and was ~90% of $^{18}$O incorporation into $\gamma$-ATP which measures total cellular energy turnover (n=5). (C) Permeabilization of cardiomyocytes for open cell-attached patch formation. i: Transmitted light image of the initial step showing a patch-pipette (1) attached to the proximal edge of a cardiac cell, and a perfusion pipette (2) approaching the distal edge of the same cell. ii-iv: Fluorescent images showing flow of a digitonin-containing solution (visualized with rhodamine; ii), and staining of the nucleus (visualized with propidium iodide; iii) upon cell permabilization; immediate withdrawal of the perfusion pipette (iv) following open cell-attached patch formation secures channel recording within a rather intact intracellular architecture. (D) $K_{\text{ATP}}$ channel activity, in an open cell-attached patch, was vigorous in the absence of ATP, and was inhibited by 2 mM, but not 0.1 mM $A_{\text{TP}}$. Yet, 0.1 mM $A_{\text{TP}}$ inhibited channel activity following application of CrP. (E) Concentration-response curves defining ATP-induced $K_{\text{ATP}}$ channel inhibition in excised (open circles), and in open cell-attached patches in the absence (open triangles) and presence (closed triangles) of 1 mM CrP. Solid curves were constructed based on the allosteric model of channel regulation (with a recalculated $k_0=10$ $\mu$M) using the highest ATP-sensitivity in the presence of CrP), in conjunction with nucleotide diffusion ($J_{\text{diff}}^{\text{ATP}}, J_{\text{diff}}^{\text{ADP}}$), ATPase ($J_{\text{ATPase}}$) and creatine kinase ($J_{\text{CK}}$) fluxes. (F) By itself CrP had no significant effect on channel opening, and required even lower ATP to block $K_{\text{ATP}}$ channels. CrP-induced channel inhibition was irreversibly antagonized by DNFB, and partially restored by purified creatine kinase (CK). Current was recorded at 21°C.

Fig. 3. Creatine kinase phosphotransfer dynamics translate cellular energetic status into membrane electrical events. (A) Hypoxia reduces CK phosphotransfer rate measured by $^{31}$P NMR in $^{18}$O-labeled hearts. (B-C) Simulation of nucleotide-dependent $K_{\text{ATP}}$ channel gating, based on the allosteric model of channel regulation integrated with nucleotide diffusional fluxes and membrane ATPase activity ($J_{\text{ATPase}}$), in the presence of vigorous creatine kinase flux ($J_{\text{ATPase}}=J_{\text{CK}}$) in normoxia (B) *versus* reduced (by 75%) creatine kinase flux ($J_{\text{ATPase}}>J_{\text{CK}}$) in hypoxia (C). While in normoxia $[A_{\text{TP}}]=[$ATP]$_{\text{sub}}$ ([$ADP]$$_{\text{sub}}=10$ $\mu$M), in hypoxia $A_{\text{TP}}<[$ATP]$_{\text{sub}}$ ([$ADP]$$_{\text{sub}}=3$ mM). Solid curves depict ATP-induced $K_{\text{ATP}}$ channel inhibition at defined $A_{\text{TP}}$ and $ADP$ sub. (D) Percent of open $K_{\text{ATP}}$ channels...
at different creatine kinase (CK) flux (180, 300, 450, 900 nmol/min/g w wt), expressed relative to creatine kinase flux in normoxia ($J_{CK} = J_{ATPase} = 1800$ nmol/min/g w wt). Each curve was constructed based on the allosteric model of channel gating, integrated with the model for nucleotide diffusion and ATPase activity (Equation 5 in Experimental Procedures), at different creatine kinase flux. (E) Shortening of action potential duration (APD), in hypoxia recorded by the monophasic action potential electrode. APD was corrected to heart rate using the modified Bazet’s formula: $APD_{90}^{*} = APD_{90} / T^{1/2}$, where $APD_{90}^{*}$ is corrected APD; $APD_{90}$, APD measured at 90% repolarization; and $T$, cardiac cycle length.

**Fig. 4. Knockout of M-CK disrupts creatine kinase-dependent control of K$_{ATP}$ channel gating.** (A) Spectrophotometric recordings of creatine kinase (CK) activity in sarcolemmal fraction from wild-type (WT) and creatine kinase-knockout (M-CK KO) hearts. Creatine kinase activity was diminished from 2.9±0.2 (n=3) in WT to 0.1±0.02 (n=3) µmol/min/mg protein in M-CK KO. (B) Concentration-response curves for creatine phosphate (CrP)-induced K$_{ATP}$ channel inhibition, at 100 µM ATP, in open cell-attached patches from WT and M-CK KO cells. Channel activity expressed relative to that measured in the absence of CrP. (C-D) K$_{ATP}$ channel recordings in WT (C) and M-CK KO (D). While in WT CrP enhanced K$_{ATP}$ channel inhibition by 100 µM ATP, in the M-CK KO the creatine kinase substrate was virtually deprived of any significant effect. Temperature was at 31°C.

**Fig. 5. Electrical instability under stress in M-CK knockout hearts.** (A) Prolonged (>20 min) metabolic challenge induced by deoxyglucose (DOG), an inhibitor of glycolysis, did not trigger electrical events in wild-type (WT) cardiomyocytes, which remain sensitive to an uncoupler of mitochondrial oxidative phosphorylation, dinitrophenol (DNP). (B) M-CK knockout (M-CK KO) hearts are highly susceptible to DOG which triggers early opening of K$_{ATP}$ channels. (C) Time course of DOG effect in WT (n=3; *open squares*) and M-CK KO (n=3; *closed circles*). While in WT there was no effect over the 17-min long DOG application, in M-CK KO the time course of DOG-induced K$_{ATP}$ channel opening was characterized by a half-maximal activation time of 16.1±0.3 min (slope: 0.8±0.1
min) obtained by the best fit of experimental data with Boltzman’s function. In A-C, channel recording was in the open cell-attached mode at 31°C. (D) Rates of action potential shortening in WT (closed circles) and M-CK KO (open squares) hearts, measured throughout DOG application fitted by linear regressions (solid lines). (E) Action potential in WT (left) and M-CK KO (right) hearts prior to (solid line) and following a 7-min long application of 5 mM DOG (dashed line).
A. % of phosphoryls oxygens replaced with $^{18}$O

B. Graph showing time vs. absorbance with CrP+ADP and Cr+ATP labeled.

C. Images with labels: i, ii, iii, iv.

D. Diagram showing ATP 2 mM and CrP 1 mM.

E. Equation: $J_{\text{diff}}^{\text{ATP}} - J_{\text{ATPase}} + J_{\text{CK}} = 0$

F. Diagram showing ATP 20 µM and CK 0.5 mg/ml.
**A**

> nmol $^{18}$O CrP/min/mg prot.

**B**

**Normoxia:** $J_{\text{ATPase}} = J_{\text{CK}}$

**C**

**Hypoxia:** $J_{\text{ATPase}} \gg J_{\text{CK}}$

**D**

% of $K_{\text{ATP}}$ channels open

**E**

Corrected AP duration

Threshold for significant shortening of AP
Coupling of cell energetics with membrane metabolic sensing: Integrative signaling through creatine kinase phosphotransfer disrupted by M-CK gene knockout
M. Roselle Abraham, Vitaliy A. Selivanov, Denice M. Hodgson, Darko Pucar, Leonid V. Zingman, Be Wieringa, Petras P. Dzeja, Alexey E. Alekseev and Andre Terzic

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