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^{31}P-NMR-measured creatine kinase reaction flux in muscle: a caveat!

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The creatine kinase (CK) isoenzyme family (E.C. 2.7.2.3), catalyzing the reversible phosphoryl transfer from phospho-creatine (PCr) to ADP, represents an important enzyme system for cellular energy homeostasis in cells with high and fluctuating energy requirements. Results obtained by cell-biological/biochemical approaches have revealed the CK system as a highly compartmentalized and complex energy buffering, distribution and regulatory network (see Wallimann *et al.*, 1989; 1992; Ventura-Clapier *et al.*, 1994; Saks *et al.*, 1994). From these results, showing distinct localizations of CK isoenzymes at subcellular sites of energy production and utilization, one may also infer compartmentation of substrates. However, the experimental results obtained by *in vivo* and *ex vivo* global ^{31}P-NMR saturation transfer measurements in muscle have so far been interpreted in a completely different way, namely, that the CK system would be in equilibrium with the substrates and behave like a solution of well-mixed enzymes (McFarland *et al.*, 1994; Wiseman & Kushmerick, 1995).

In these recent articles, Dr Kushmerick and colleagues reported that "the CK chemical kinetics in the intact cell is adequately described in terms of a well-mixed solution of enzyme, substrate and products etc" (McFarland *et al.*, 1994: 1922) and that "effects of compartmentalization, however important for certain functions, are negligible with respect to total cellular bioenergetics and metabolism" and "that thermodynamic characteristics of the cytosol can be predicted as if these metabolites were freely mixing in solution" (Wiseman & Kushmerick, 1995: 12437).

In the first publication, an ^{31}P-NMR saturation transfer study measuring CK flux in resting and stimulated *ex vivo* perfused soleus muscle was presented (McFarland *et al.*, 1994). The authors observed that in this muscle the creatine kinase (CK) reaction flux did not increase during steady-state stimulation over a ten-fold range of ATPase rates. This is contrary to what obligatory phosphocreatine (PCr) shuttle models (see Wallimann *et al.*, 1992 and references therein) would predict, if one assumes that the work-load imposed on the muscle was large enough to challenge the CK system which has a vast overcapacity compared to the myofibrillar ATPase (Ventura-Clapier *et al.*, 1994). A closer look at the data presented reveals that the CK activity measured by an enzymatic assay in the contra-lateral soleus would correspond to a forward flux of 28 mM s^{-1}, which is about ten times higher than the flux actually measured by spin transfer NMR *in vivo* (approximately 2.5–3.0 mM s^{-1}) (McFarland *et al.*, 1994). This result suggests that *in vivo* reaction rates can differ significantly from rates measured *in vitro*. In this specific case, only about 10% of the CK reaction flux expected from enzymatic measurements is actually seen by the *in vivo* NMR technique and this reaction flux at rest does not seem to increase with increasing work load. To explain the discrepancy between enzymatic reaction rates and *ex vivo* NMR flux measurements, the authors suggest that CK is inhibited by chloride and bicarbonate ions present in the perfusion solution which may form an anion-stabilized quaternary dead-end-enzyme complex composed of CK, Cr, MgADP and the respective anion. However, no actual measurements of this purported enzyme inhibition were reported, even though this could have been done very easily with the controlateral muscle of the same animal. Much of the interpretation of the results was then based on this assumption and on a computer simulation of such a CK-inhibition (see Fig. 8, McFarland *et al.*, 1994).

On the other hand, the knowledge on the biochemistry and cell biology of CK isoenzymes accumulated over the past two decades clearly reveals that the CK/Cr-system is highly compartmentalized on a cellular and subcellular level

(Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Wegmann *et al.*, 1992; Wallimann & Hemmer, 1994; Ventura-Clapier *et al.*, 1994; Saks *et al.*, 1994; Kraft *et al.*, 1996).

In muscle, some of the CK is bound in an isoenzyme-specific fashion at sites of energy utilization or production, e.g. at the myofibrillar M-band (where muscle-type cytosolic M-CK is functionally coupled to the acto-myosin ATPase), the I-band (where it is functionally coupled to glycolytic enzymes which are also located at the actin-filament region), at the sarcoplasmic reticulum (in tight conjunction with the Ca^{2+}-ATPase), the sarcolemma (connected to the Na^{+}/K^{+}-ATPase), as well as at the mitochondria (where the mitochondrial CK isoenzyme, Mi-CK, is functionally coupled to oxidative phosphorylation) (see Wallimann *et al.*, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994; Wallimann & Hemmer, 1994; and references therein).

While an effort can be made to model a uniformly mixed functional system for CK (McFarland *et al.*, 1994; Wiseman & Kishmerick, 1995), such a construct is neither supported by modern concepts of cell biology in general, nor does it seem to apply for muscle in specific, because there is plenty of evidence that sarcomeric muscle is a very highly organized tissue showing paracrystalline subcellular structures where many enzymes are localized exclusively at distinct subsarcomeric regions to fulfil a regionally specific metabolic function (Wegmann *et al.*, 1992; Kraft *et al.*, 1995, 1996). This enzyme localization holds true for the CK isoenzyme system which is highly compartmentalized and subcellularly organized in muscle and other tissues (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Wallimann & Hemmer, 1994). Depending on the muscle fibre type, significant amounts of this enzyme are more or less firmly bound to subcellular structures or, as is the case with mitochondrial Mi-CK, are situated exclusively within mitochondria (Wyss *et al.*, 1992; Wyss & Wallimann, 1992), where Mi-CK octamers, based on their molecular structure (Schnyder *et al.*, 1990, 1994), are likely to be involved in metabolic channelling of high-energy phosphates (Wyss & Wallimann, 1992; Saks *et al.*, 1994).

Considering these biological complexities, one is tempted to suspect that the experimentally reported near-equilibrium situation for the entire CK system, as observed by *in vivo* ^{31}P-NMR measurements (McFarland *et al.*, 1994; Wiseman & Kushmerick, 1995), may be a consequence of certain limitations of the rather global NMR method and/or may be due to a peculiar unexpected *in vivo* behaviour, in NMR terms, of the CK system itself. As a matter of fact, strong evidence for the latter has now been produced rather serendipitously by investigating the CK flux with ^{31}P-NMR methods in skeletal muscle of transgenic mice which do no longer express enzymatically active cytosolic muscle-type M-CK (vanDeursen *et al.*, 1993) and in mice which show graded expression of M-CK activity in their muscles (vanDeursen *et al.*, 1994). First of all, it was found that mice lacking M-CK activity quite unexpectedly still utilized PCr as an energy source, most likely by derouting PCr via Mi-CK as a consequence of metabolic adaptation, but, strangely enough, no CK flux could be measured by the same ^{31}P-NMR techniques (vanDeursen *et al.*, 1994) as applied by McFarland and colleagues (1994). A CK flux could only be measured in those mice which expressed at least 34% or more of the normal wild type levels of M-CK activity (vanDeursen *et al.*, 1994). Between 34% and 50% of wild-type M-CK activity levels, the measured CK flux sharply increased to 100% of the normal ^{31}P-NMR-flux measured in wild-type mice, while, surprisingly, no further increase in CK flux was observed in mice expressing from 50% to 100% of wild-type CK levels (vanDeursen *et al.*, 1994). Thus, it seems that a large proporton of CK in the intact tissue is not amenable to CK flux measurements by current *in vivo* saturation transfer ^{31}P-NMR methods and protocols.

Since, according to our estimates and, depending on the muscle type, some 30–50% of total cellular CK are bound to subcellular structures and/or are contained within the mitochondria (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994), it seems very likely that this very bound fraction of CK is indeed 'silent' in *in vivo* and *ex vivo* CK flux measurements by ^{31}P-NMR. This is in agreement with the interpretation given by vanDeursen and colleagues (1993, 1994), who suggested that an NMR-measurable CK flux due to the appearance of strictly soluble, cytosolic M-CK appears only after saturating the cellular binding sites with CK (e.g. at the myofibrils, the SR and the plasma membrane (Wallimann *et al.*, 1992; Wegmann *et al.*, 1992; Ventura-Clapeir *et al.*, 1994)) by more than 34% of normal M-CK levels (vanDeursen *et al.*, 1994). In other words, most of the CK fluxes measured in intact muscle by *in vivo* or *ex vivo* saturation or spin transfer ^{31}P-NMR may be derived from the action of a certain pool of soluble cytosolic CK (mainly the fraction between 34–50% of normal CK activity expression levels), whereas a significant other soluble CK pool (e.g. the fraction between 50–100% of normal CK activity expression), as well as the bound enzyme and/or the fraction of CK which is, for example, functionally coupled to an ATPase (see Wallimann *et al.*, 1992) may not be seen by such NMR measurements. According to our PCr-circuit model (Wallimann *et al.*, 1992; Ventura-Clapier *et al.*, 1994; Saks *et al.*, 1994), an equilibrium situation is proposed for cytosolic CK. Since in muscle, this

enzyme is present in a large excess, it is thought to equilibrate fully ATP/ADP and PCr/Cr pools (Wallimann *et al.*, 1992). The bound and/or functionally coupled CKs (to ATP-regenerating or ATP-utilizing systems) may behave quite differently, with the CK fluxes through the bound enzyme increasing with higher muscle performance. Thus, it seems likely that the NMR measurements cannot tell us what is really going on at those subcellular sites where CK is bound and functionally coupled to ATPases or firmly integrated into a mitochondrial multi-enzyme channelling complex allowing for vectorial transport of high energy phosphates (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Wegmann *et al.*, 1992; Wyss & Wallimann, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994).

These observations indicate that NMR-flux measurement obtained with organs or tissues cannot necessarily be extrapolated to the 'real events' taking place at the cellular or even subcellular level and therefore should be interpreted with caution. The trend at very high work load towards decreasing rather than increasing CK fluxes (see Fig. 6 in the work of McFarland *et al.*, 1994) would indicate that, under the paradigm elaborated above, the proportion of cytosolic versus bound CK may shift under heavy work-load towards the bound population. This interpretation suggests that the apparent CK flux may be a larger, but NMR-hidden flux through bound, functionally coupled CK (Wallimann, 1994). Such additional complications, i.e. work-load-dependent reversible associations of enzyme systems with the corresponding subcellular structures (e.g. myofilaments), recently shown to take place upon low-frequency stimulation of muscle (Parra & Pette, 1995), may thus also influence the reaction flux measurements by NMR and therefore would have to be taken into consideration as well.

Based on these arguments, the data of McFarland and colleagues (1994) would mean nothing more than that the CK flux through the strictly soluble, cytosolic M-CK pool does not change with increasing work-load imposed on the muscle, which, as mentioned above, is in full agreement with one of the main functions proposed for soluble, cytosolic CK, which shows much higher specific activity than the muscle ATPases, as a temporal metabolic buffer and capacitor within the PCr-circuit model (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Ventura-Clapier *et al.*, 1994; Saks *et al.*, 1994). However, according to the interpretation of McFarland and colleagues (1994) and Wiseman and Kushmerick (1995), the NMR results would imply no important role for Mi-CK in energy transport or spatial buffering, a function which has been postulated on the basis of well documented experiments with isolated mitochondria and skinned muscle fibres (see Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994). If the PCr-circuit hypothesis, incorporating both a temporal and spatial buffering function, as well as a regulatory function of the CK system, holds true (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994; Zeleznikar & Goldberg, 1991; Zeleznikar *et al.*, 1995), with metabolic wave propagations facilitated by the CK system (Wallimann *et al.*, 1989; Saks *et al.*, 1994), one would expect very high bi-directional phosphoryl transfer events back and forth between PCr and γ-ATP phosphate, facilitated by the action of CK. This exchange event, also involving the adenylate kinase system (Zeleznikar & Goldberg, 1991; Zeleznikar *et al.*, 1995), and therefore affecting β-phosphate transfer as well (Zeleznikar & Goldberg, 1991; Zeleznikar *et al.*, 1995), is likely to obscure the actual NMR flux measurements. In addition, an exchange between γ-ATP phosphate and inorganic P_i, which is indicated to take place in soleus (see Fig. 3 of McFarland and colleagues (1994), Dr Heerschap, Nijmegen, personal communication), would also have to be considered.

The conclusions made by Kushmerick and colleagues may mislead some readers to assume that the CK/PCr-function is mainly that of a temporal buffer (Meyer *et al.*, 1984) and that, therefore, spatial buffering or vectorial transport of high-energy phosphates, facilitated by precisely localized CK isoenzymes, as well as other regulatory functions of the CK system (Wallimann *et al.*, 1992; Wyss *et al.*, 1992), based on the specific subcellular compartmentation of the CK system, could be neglected or dismissed entirely. This development is unfortunate, for it comes at a time when CK is on the verge of returning into the limelight. The possibility of its structure determination by X-ray diffraction (Schnyder *et al.*, 1990; Fritz-Wolf *et al.*, 1996) and the rather exciting new findings concerning CK involvement in viral replication (Lillie *et al.*, 1994), cancer progression (Lillie *et al.*, 1993) and in certain signalling cascades (Kuzhikandathil & Molloy, 1994) and cell cycle regulation (Martin *et al.*, 1994) are important new developments.

Considering the arguments listed above, the statements made by Kushmerick and colleagues are somewhat surprising since they also fail to take into account the recently reported quite anomalous behaviour, in NMR terms, of creatine in muscle, i.e. nonisotropic spectral contributions of protons from the methyl group of creatine (Kreis & Boesch, 1994). The latter observation led Kreis and Boesch to postulate a liquid-crystal-like behaviour of compounds as small as creatine in muscle, most likely due to the highly organized structure of this tissue. As a matter of fact, new data measuring by laser confocal microscopy the diffusion and equilibration

of different fluorescently-labelled compounds in chemically skinned skeletal muscle fibres revealed that the time for equilibration of these compounds within the myofilament lattices cannot simply be predicted from simple diffusion parameters (see Kraft *et al.*, 1995 and references therein concerning the diffusion of small molecules within cells). Furthermore, evidence for subcellular compartmentation of ATP in much smaller cells than muscle comes from studies with insulinoma cells of the pancreas which show global ATP contents of 2–8 mM. Since the ATP-gated K^+-channels of these cells, the closure of which is vital for insulin release, close at higher concentrations of ATP than 100 μM, they could never open if there were no mechanisms for compartmentation of nucleotides, which in these cells seems to be facilitated by the CK system (Gerbitz *et al.*, 1996). In full accordance with this, Goldberg and colleagues, using ^{18}O-labelling of metabolite phosphoryls, were able to demonstrate a remarkable degree of metabolite compartmentation (Zeleznikar & Goldberg, 1991), as well as an interdependent, complementary connection of the CK system with the adenylate kinase system in muscle (Zeleznikar *et al .*, 1995, and references therein). A rough comparison of these ^{18}O-labelling data with the ^{31}P-NMR flux measurements of McFarland and colleagues (1994) shows a rate of exchange (CK flux) which is approximately 20–30-fold different!

In conclusion, the new and unexpected situation concerning the obvious anomalies with the measurability of *in vivo* CK fluxes by ^{31}P-NMR methods seen in transgenic CK mice (vanDeursen *et al.*, 1993, 1994) should stimulate re-thinking of the global interpretation of NMR results (McFarland *et al.*, 1994; Wiseman & Kushmerick, 1995). An inability to observe the flux through the bound or compartmentalized CK fractions does not necessarily mean that there is no such flux to occur. Thus, the complex PCr-circuit system, mainly postulated on biochemical and cell biological grounds, may impose some formidable NMR problems to be solved. The present trend, however, to interpret the ^{31}P-NMR flux measurement data by the simplest model possible (McFarland *et al.*, 1994; Wiseman & Kushmerick, 1995) may lead to a situation where the global NMR-based view of a cell, i.e. as a "well mixed bag of enzymes", is utterly simplified and far removed from the complex reality of modern cell biology. New approaches, e.g. high-resolution microcoil NMR for mass-limited, nanoliter-volume samples (Olson *et al.*, 1995) may help to reach cellular, time-resolved dimensions, and new outlooks may hopefully make possible the integration of the present knowledge about the biochemical and structural characterization of the CK isoenzymes. The interpretation of results obtained from *in vivo* ^{31}P-NMR experiments must take into account the facts concerning the subcellular localization and manifold functions of CK isoenzymes within different cells of fluctuating high energy requirements (Wallimann & Hemmer, 1994), as well as the documented structural and functional coupling of CKs to other enzyme systems, channels and transporters (Wallimann *et al.*, 1992; Wyss *et al.*, 1992; Wyss & Wallimann, 1992; Saks *et al.*, 1994; Ventura-Clapier *et al.*, 1994; Zeleznikar & Goldberg, 1991; Brdiczka *et al.*, 1994; Zeleznikar *et al.*, 1995). It is to be hoped that, by this way, a concept for CK function valid all the way from the molecular to the whole organ level can be put forward and thus constructive solutions of these complex issues can be proposed in the future.

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