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CHEMISTRY

Ultra performance liquid chromatography analysis of adenine nucleotides and creatine derivatives for kinetic studies

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Abstract. A rapid method for simultaneous quantification of compounds participating in energy metabolism of cardiac muscle cells (creatine (Cr), phosphocreatine (PCr), ADP, and ATP) is described where a conventional ion-pair reversed phase HPLC separation has been improved by introducing the method based on the recently developed ultra performance liquid chromatography (UPLC) technique. In the 0.005–1 mM concentration range, the calibration curves for Cr, ADP, and ATP as pure standard compounds were fitted by the polynomic relationship $y = y_0 + ax - bx^2$ at a high confidence level ($R^2 > 0.999$ in all cases) and that for PCr by a simple linear relationship with $R^2 > 0.998$. The method was applied for the study of the kinetics of PCr production by permeabilized cardimyocytes due to the cellular oxydative phosphorylation reactions. The determined steady-state levels of ADP and ATP as well as the rate of PCr production in different conditions can be used for the verification of the results of mathematical modelling of cardiomyocyte functioning.

Key words: analytical biochemistry, ultra performance liquid chromatography (UPLC), cardiac energy metabolism, creatine kinase.

INTRODUCTION

The aim of the study was to develop a sufficiently rapid, high-throughput HPLC method for simultaneous determination of high-energy phosphorus compounds in biological samples that would be suitable for the analysis of ATP resynthesis in cardiac myocytes and, specifically, would be applicable for the elucidation of details of the creatine kinase (CK) reaction:

$$\text{Creatine} + \text{MgATP}^{2-} \leftrightarrow \text{Phosphocreatine}^{2-} + \text{MgADP}^{-} + \text{H}^{+} \quad (1)$$

in this process. The CK reaction catalysed by its mitochondrial isoenzyme MtCK in the mitochondrial intermembrane space is directed towards the synthesis of another high-energy phosphorus compound, phosphocreatine (PCr), which is liberated into the cytoplasm through the mitochondrial voltage-dependent anion selective channels (VDAC) leaving another reaction product, ADP, for further ATP resynthesis in the mitochondrial matrix.

Separation of adenine nucleotides, creatine (Cr) compounds, and other metabolites extracted from biological samples has been a routine task for decades, and a large variety of HPLC procedures have been proposed for performing it (cf. Brown et al., 1980; Grune and Siems, 1993 for review). In earlier studies ion-exchange HPLC columns have been used but found to be inconvenient due to their poor stability and/or long elution times required for re-equilibriation of the columns after applying the salt gradient. In parallel, common reversed-phase C_{18} columns have been used applying different isocratic or combined isocratic–gradient elution modes. HPLC methods applying the reversed phase technique, where the eluating buffer solution is supplemented by a large hydrophobic cationic, typically a tetrabutylammonium (TBA) compound, were probably first used for the separation of nucleotides by Juengling and Kammermeier (1980),

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who applied two separate isocreatic runs in different conditions to resolve Cr, PCr, and adenine nucleotides.

A number of similar HPLC separation protocols have further been described, which applied different elution modes. A selection of these protocols used to resolve Cr derivatives and nucleotide-related compounds in samples extracted from cardiac tissue or skeletal muscle is presented and analysed in greater detail in the Materials and Methods section. Briefly, elution of Cr and PCr (as well as creatinine, see Fürst and Hallström, 1992) is performed in isocratic mode in the presence of TBA followed by elution of nucleotides in another buffer solution containing organic solvent ('step gradient'), or in continuous organic solvent gradient mode.

In the ion-pair HPLC method, the separation of (negatively) charged compounds is expectedly achieved due to (1) the modification of the column's surface by reversible binding of large hydrophobic cations, e.g., TBA; and (2) the binding of the complex of the cation with oppositely charged species formed in the mobile phase to the hydrophobic stationary phase (see, e.g., Grune and Siems, 1993). Nevertheless, for nucleotides as well as Cr and PCr only the first mechanism seems to hold as it was generally accepted years ago (Smith and Alberty, 1956a, 1956b) that TBA cation does not associate with phosphate-containing ligands in water solutions. This statement was confirmed in a more recent ^{31}P NMR study (Cecconi et al., 2002) showing that TBA could be used as 'internal reference cation' (cf. Smith and Alberty, 1956a, 1956b), which did not induce any shifts in the NMR spectra of PCr in conditions where a weak association of PCr with Na^+ ($\log K_{ass} = -0.5 \pm 0.2$) as well as K^+ ions ($\log K_{ass} = -0.3 \pm 0.2$) could be readily determined. Accordingly, positively charged hydrophobic reversed phase column surface covered with diffuse layer(s) of hydrophobic cations, thus supplying combined hydrophobic and electrostatic interactions, seems to be the main separating factor for hydrophilic Cr and PCr as well as more hydrophobic nucleotides.

MATERIALS AND METHODS

Chemicals

TBA bisulphate puriss p.a. for ion pair chromatography (Fluka), Na_2HPO_4 (Fluka), and acetonitrile (Rathburn) were HPLC grade reagents. Soya bean trypsin inhibitor (STI), fatty acid free bovine serum albumin (BSA), pyruvate kinase (PK), leupeptin, ATP, ADP, PCr, ethylene glycol-bis(2-aminoethyl)-N,N,N′N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) were from Roche, N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), Cr, and other reagent grade chemicals used were from Fluka or Sigma.

Methods

Isolation of cardiac myocytes

Adult cardiomyocytes were isolated after perfusion of the rat heart with collagenase using the adaptation of the technique described previously (Saks et al., 1991). Briefly, male Wistar rats (300–350 g) were anaesthetized by 200 mg/kg pentobarbital with the addition of 500 U of heparin. The heart was quickly excised preserving a part of aorta and placed into washing solution (WS) of the following composition: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 120 mM sucrose, 10 mM Cr, 20 mM taurine, and 21 mM BES, pH 7.1 at 25 °C. All solutions were saturated with oxygen. The heart was cannulated and washed with WS at a flow rate of 15–20 mL/min for 5 min. The collagenase treatment was performed by switching the perfusion to recirculating isolation medium (IM), which included 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM Cr, 20 mM taurine, 10 mM PCr, 2 mM pyruvate, and 21 mM HEPES, pH 7.1, supplemented by 0.03 mg/mL Liberase Blendzyme I (Roche) at a flow rate of 5 mL/min for 50 min. Switching the perfusion to the initial WS for 2–3 min terminated the collagenase treatment. After the digestion, the heart was washed with the IM during 2–3 min and transferred into the IM containing 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM STI, and 2 mg/mL BSA. The cardiomyocytes were then gently dissociated by forceps and pipette suction. The cell suspension was filtered through a crude net and transferred into a tube for sedimentation. The initial supernatant was discarded and the calcium-tolerant contracting cells were allowed to freely sediment. The procedure was repeated twice. Cardiomyocytes were gradually transferred from IM containing 20 μM free calcium into (nominally calcium-free) Mitomed. Finally, the cardiomyocytes were washed 5 times with the same solution containing BSA and the proteinase inhibitors as described above. Isolated cells were stocked in 1–2 mL volume and stored on ice during further experiments. Isolated cardiomyocytes contained 80–90% of rod-like cells when observed under a light microscope. The cells were quantified by protein content determined with the Pierce BCA Protein Assay Kit according to the manufacturer's instructions.

CK reaction

The reaction was performed in the conditions suggested for kinetic studies on the respiration of permeabilized cardiomyocytes (Kuznetsov et al., 2008) in the medium

containing 0.5 mM EGTA, 3 mM Mg-acetate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol (DTT), 5 mg/mL BSA, 5 mM glutamate, and 2 mM malate at pH 7.1 (Mitomed) at 25 °C by withdrawing aliquots of 100 µL at 1.5 (or 3), 6, and 10 min.

Sample preparation

The withdrawn 100 µL aliquots were added to 200 µL ice-cold 1 M $HClO_4$ solution, immediately supplemented with 5 µL of 100 mM EDTA, and neutralized with 210 µL of 0.952 M KOH for 0.5–1 min. The samples were held on ice for additional 10–15 min for proper precipitate formation and centrifuged at 4 °C and 16 000 *g* for 2–3 min. The supernatants were immediately frozen (–40 °C) and analysed within 5–6 h after defrosting holding the samples in the thermostatted autosampler chamber at 4 °C.

UPLC chromatography

Separations of Cr, PCr, and adenine nucleotides were performed on a 2.1 × 100 mm ACQUITY UPLC HSS T3 column packed with 1.7 µm particles (Waters) by recording the optical density simultaneously at 210 nm for Cr and PCr and at 254 nm for adenine nucleotides. Sample volumes of 10 µL were injected by an autosampler. The mobile phase consisted of buffer A (20 mM Na_2HPO_4, 3 mM TBA bisulphate) and buffer B (200 mM Na_2HPO_4, 10% (v/v) acetonitrile, 0.3 mM TBA bisulphate), both adjusted to pH 5.0 with 2 M phosphoric acid and filtered through a 0.2 µm membrane filter. The elution was performed at a flow rate of 0.4 mL/min in buffer A for 2 min followed by 1 : 1 gradient elution with buffers A and B up to 8.5 min and additionally with buffer B up to 10 min. After the analysis the column was re-equilibriated by washing for 1 min with water and 9 min with buffer A, thus resulting in the total recycling time of 20 min. The column was cleaned by washing with 80% methanol after every series of experiments, which typically included 25–30 chromatographic runs. Calibration stock solutions (0.1 M) were prepared in 0.2 M Na_2HPO_4 at pH 7.0 and stored at –40 °C for not more than 2–3 days in order to minimize PCr and ATP degradation. Alternatively, calibration solutions were prepared in supernatant solutions obtained as described above (see 'Sample preparation').

RESULTS AND DISCUSSION

Previous studies and UPLC

The methods used for simultaneous separation of Cr compounds and adenine nucleotides generally include two types of protocols: (1) isocratic elution of these compounds in an organic solvent-free buffer followed by stepwise switch to another buffer contaning acetonitrile or methanol and (2) typical gradient elution method where Cr derivatives are eluted in isocratic conditions and adenine nucleotides by organic solvent gradient elution. In both types of protocols the choice of pH and ionic strength of the buffer solution and TBA concentration is highly variable in different studies (Table 1). Reportedly, the choice of the two isocratic elution modes gives shorter total elution times; however, the time required for full re-equilibriation of the column, i.e. the recycling time, is typically not specified (except, e.g., Cordis et al., 1988). The recycling time is typically specified in gradient elution protocols, which give recycling times of about 50 min. In general, while attempts for the separation of hydrophilic Cr derivatives (including creatinine, cf. Fürst and Hallström, 1992) could face difficulties in proper peak resolution, the separation of nucleotides by ion-pairing HPLC could be achieved nearly in all conditions. Moreover, care should be taken to avoid too tight binding of the nucleotides in the column rather than make efforts to achieve properly resolved peaks for the species: it has been shown that dozens of nucleotide metabolites could be resolved in a single run (see Grune and Siems, 1993 for review).

Introduction of the UPLC methods that explore a new generation of small-diameter particles (1.7 µm in the present case) along with the pertinent equipment ensured higher resolution and sensitivity and, importantly, an impressive reduction in analysis time (up to 10-fold in some cases, see Wilson et al., 2005) due to high, up to 1000 atm, pumping pressures used. Looking for a prototype for our UPLC protocol we proceeded from that proposed by Vives-Bauza and colleagues (Vives-Bauza et al., 2007). In this procedure, Cr and PCr are separated in a low ionic strength phosphate buffer (cf. Table 1) and nucleotides by gradient elution in high ionic strength phosphate buffer, which has a moderate acetonitrile concentration at a very low TBA concentration. The use of sodium salts instead of potassium salts used in the majority of other studies (Table 1) is also preferable due to the lower ability of Na^+ to the complex formation with PCr (Smith and Alberty, 1956b; Cecconi et al., 2002) as noted in Introduction. A representative chromatogram of standard compounds obtained in selected conditions is presented in Fig. 1 showing the elution of Cr close to the void volume of the column, isocratic elution of PCr followed by gradient elution of ADP and ATP taking in total 10 min.

Table 1. Conditions for ion-pair HPLC separation and quantification of creatine derivatives and adenine nucleotides extracted from cardiac muscle preparations. A selection of reports

Reference	pH[a]	Elution conditions[b]	Elution mode[c]	Elution time, min[d]	Particle size, column size, column specification
Juengling and Kammermeier, 1980	3.0 5.8	(A) 2.35 mM KH_2PO_4 (0.2%), 15 mM TBA phosphate (0.08%) (B) 48 mM KH_2PO_4 (0.65%), 8.8 mM TBA phosphate (0.65%), 21.8% AcN	Iso Iso	ca. 5 ca. 8	? μm, 4 × 250 mm, Lichrosorb, Merck C8/C18
Sellevold et al., 1986	6.25	(A) 215 mM K_2HPO_4, 2.3 mM TBA bisulphate (B) Buffer (A) + 3.5% AcN	Iso Iso	10	5 μm, 150 mm, Supelcosil
Sanduja et al., 1987	6.7	30 mM K_2HPO_4, 15 mM TBA bisulphate, 19% AcN	Iso	25	5 μm, 4.6 × 250 mm, Ultrasphere ODS
Cordis et al., 1988	5.8	(A) 48 mM KH_2PO_4, 1 mM TBA phosphate (B) Buffer (A) + 20% AcN	Iso Iso	4 10 Total 35	4 μm, 5 × 100 mm, Nova-Pak C18
Ally and Parks, 1992	6.0	(A) 35 mM K_2HPO_4, 6 mM TBA bisulphate, 125 mM EDTA (B) Buffer (A) and AcN (1 : 1)	Iso Iso	10 10 + 5	5 μm, 4.6 × 250 mm, Supelco LC18-T or 5 μm, 3.9 × 150 mm, Nova-Pak C18
Scott et al., 1992	6.5	(A) 100 mM K_2HPO_4, 0.01 mM TBA phosphate, (B) Buffer (A) + 40% CH_3OH	Iso Iso	8 7	3 μm, 4.6 × 70 mm, Ultrasphere ODS
Botker et al., 1994	6.0 6.0	(A) Cr compounds: 20 mM K_2HPO_4, 2.3 mM TBA bisulphate (B) Nucleotides: 60 mM K_2HPO_4, 11 mM TBA bisulphate, 25% CH_3OH	Iso Iso		5 μm, ? × 150 mm, Supelcosil LC 18-DB
Karatzaferi et al., 1999	5.3 6.5	(A) 14.7 mM KH_2PO_4, 1.15 mM TBA phosphate (B) 215 mM KH_2PO_4, 2.3 mM TBA phosphate, 2% AcN	Iso Iso	ca. 12	5 μm, 4 × 125 mm, LiChrosphere HP
Fürst and Hallström, 1992	5.5	(A) 100 mM Na_2HPO_4, 5.9 mM TBA bisulphate (B) Buffer (A) and AcN (75 : 25)	Iso 0–17%	5 22 Total 47	5 μm, 4 × 250 mm, Hypersil ODS
Bernocchi et al., 1994	6.0 5.5	(A) 100 mM KH_2PO_4, 5 mM TBA bisulphate, 2.5% AcN (B) Buffer (A) + 25% AcN	Iso 0–11% 11–100%	3 2 25	3 μm, 4.6 × 150 mm, Supelchem C18
Vives-Bauza et al., 2007	5.0 4.0	(A) 25 mM NaH_2PO_4, 100 mg/L TBA sulphate (B) 200 mM NaH_2PO_4, 100 mg/L TBA sulphate, 10% AcN (0.3 mM TBA)	Iso 0–10%	3	3 μm, 4.6 × 150 mm, Waters YMC C18
Volonté et al., 2004	?	215 mM K_2HPO_4, 2.3 mM TBA sulphate, 4% AcN	Iso	5	5 μm, 4 × 250 mm, LichrocartRP 18
Williams et al., 2008	6.0 5.5	(A) 100 mM K_2HPO_4, 5 mM TBA sulphate, 2.5% AcN (B) Buffer (A) + 25% AcN	Iso 2.5–11% 11–25%	3 2 25 Total 50	3 μm, 4.6 × 150 mm, Supelchem C18 or ? μm, 4 × 250 mm, Hypersil ODS

[a] pH values are given as specified. Note that possible changes in pH induced by addition of organic solvent are not reported in some studies. [b] AcN = acetonitrile. [c] Iso = isocratic; details of organic solvent gradient are specified. [d] In some cases, noted as e.g. 'ca. 5', the elution times were estimated from original figures. For gradient elutions limiting organic solvent concentrations are indicated. Total column recycling times are presented when originally specified.

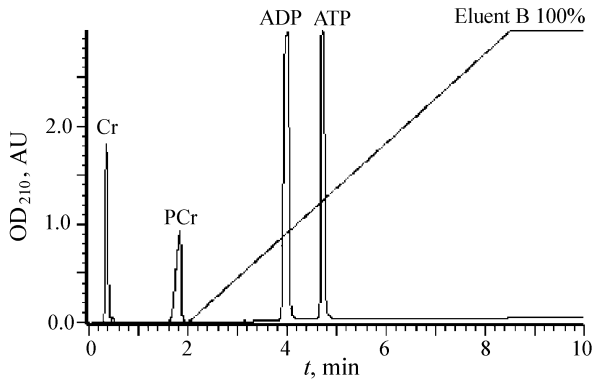

Fig. 1. Representative chromatogram for standard compounds. The retention times for the reagents were, in minutes, 0.63 (Cr), 1.70 (PCr), 6.95 (ADP), and 7.29 (ATP), all typically within ±0.01 min. The gradient of acetonitrile is indicated, recording was performed by optical density at 210 nm in the present case.

Calibration curves for standards and actual calibration

It is typically stressed in HPLC studies that the peak area versus concentration plots give linear relationships over a certain (large) concentration range of the standard compounds. In the present conditions this assumption did not fully hold and the shape of the curves tended to depend on the structure of a particular compound (Fig. 2). The peak area versus concentration plot for Cr (Fig. 2a), which is eluted close to the void volume of the column (Fig. 1), exhibits a slight curvature and could be best fitted by the polynomic relationship $y = y_0 + ax - bx^2$. A similar curvature can be observed for ADP and ATP (Fig. 2b). At the same time, the data for PCr gave a perfectly straight line (Fig. 2a) with 1.9% standard deviation in the slope value in the same concentration range (Table 2). These differences most likely demonstrate an excellent quality of the whole UPLC equipment rather than have any substantial practical meaning provided that the calibration has been properly used: typically, the observed $R^2 > 0.99$ in all cases regardless of whether the polynomic or linear equation was used for data fitting (Table 2). The difference between the two calibration equations is best visualized as an uneven distribution of the residuals from linear relationships as presented in Fig. 3, where the residuals are presented as percentage of actually determined peak areas. Evidently, deviations of the data from simple linear calibration plots observed at low concentrations make a minor contribution into the R^2 value but could substantially (more than 2-fold) distort the results. In this situation we preferred to perform standardizations in a shorter concentration range (typically 4 points and 4-fold change in the concentration), which covers the concentrations actually observed in kinetic studies, thus enabling to apply simple linear relationships for calibration. Actual calibration solutions were made up in the solution obtained after precipitation of the cells and proteins as described in Materials and Methods.

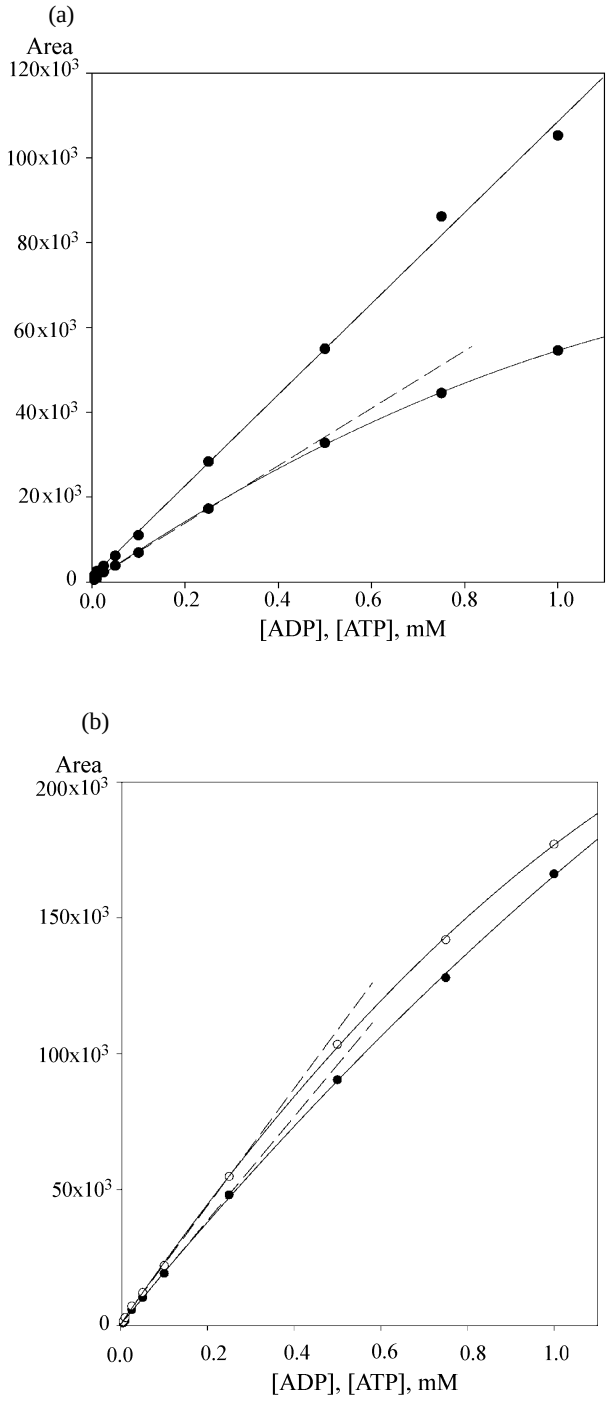

Fig. 2. Calibration curves for standard compounds in the cell-free medium. (a) Phosphocreatine (upper straight line) and creatine. The dashed line depicts the initial slope of the curve for creatine. (b) ADP (filled cycles) and ATP.

Table 2. Parameters of the standard curves for pure compounds

Compound	y_0	$a \times 10^{-5}$	$b \times 10^{-5}$	R^2
Cr	143.8 ± 158.8	0.7442 ± 0.0116(1.6%)	0.2001 ± 0.012(6.1%)	0.99983
	1458 ± 863	0.5598 ± 0.0188(3.4%)		0.99214
PCr	437.6 ± 974.5	1.185 ± 0.071(6.0%)	0.1214 ± 0.0747(61.5%)	0.99824
	1235 ± 936	1.073 ± 0.020(1.9%)		0.99747
ADP	459 ± 472	1.933 ± 0.035(1.8%)	0.2824 ± 0.0361(12.8%)	0.99983
	2313 ± 1262	1.673 ± 0.028(1.6%)		0.99810
ATP	5667 ± 433	2.311 ± 0.032(1.4%)	0.5481 ± 0.0332(6.1%)	0.99988
	4166 ± 2363	1.805 ± 0.052(2.9%)		0.99432

Note: The curves were calculated from the peak area versus concentration plots for the 0.005–1 mM concentration range (9 points) according to the polynomic equation $y = y_0 + ax - bx^2$ (upper rows) and linear equation $y = y_0 + ax$ by the least squares' fit. Standard errors and their percentages in coefficients *a* and *b* are shown.

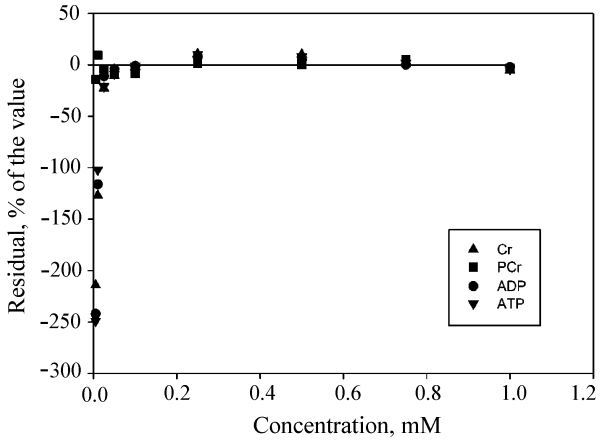

Fig. 3. Distribution of the residuals of the determined values from linear peak area versus concentration relationships. The data represent residuals as percentage of the measured values.

Sample preparation

The samples were prepared by stoping the reaction by the standard method of precipitation by perchloric acid, neutralization of the solution with KOH, and removal of the $KClO_4$ and protein precipitates by centrifugation (cf. Brown et al., 1980; Grune and Siems, 1993) as described in Materials and Methods. The risk factors for the procedure are (1) instability of PCr as well as Cr at (highly) acidic pH values (see, e.g. Carter and Müller, 1990; Botker et al., 1994), (2) improper $KClO_4$ precipitation, and (3) residual adenylate kinase (AK) activity, which cannot be fully and irreversibly inhibited by the applied acid treatment. The first two risk factors suggest that it is advisable to avoid double centrifugation steps (centrifugation of acid precipitate followed by centrifugation of $KClO_4$ precipitate after neutralization), to perform acid precipitation and neutralization in a few minutes and centrifuge the pellet at least after 10 min for proper precipitate formation (cf. Botker et al., 1994) as described in Materials and Methods. Residual AK activity could be suppressed by a powerful AK inhibitor, diadenosinepentaphosphate (our unpublished observations); however, its use is inconvenient in the present case since the compound binds to the column too strongly thus prolonging the separation cycle due to the need for harsh conditions for its wash-out. Another simple and efficient way to suppress the AK activity is the use of EDTA as a strong Ca^{2+}- and Mg^{2+}-binding agent since it is well known that ATP-converting enzymes typically require the binding of both ATP and Mg^{2+} for their activity, which, in turn, is often calcium-dependent. Surprisingly, to our knowlege this effect of EDTA (and EGTA) has been explored only in a single, relatively early study (Ally and Parks, 1992).

Sample run

The samples were run in isocratic mode in a low ionic strength Na phosphate buffer and moderate TBA concentration (3 mM) followed by gradient elution in a high ionic strength buffer solution containing a low TBA concentration (0.3 mM) and acetonitrile in 10 min as described in Materials and Methods. Further the column was flashed with water for 1 min and equilibrated with buffer A for 9 min. Shorter re-equilibrium times, e.g. 5 min, tended to result in shifts in retention times although the peak resolution and calculated peak areas remained unaffected. Thus, despite all advantages of the UPLC method, the total recycling time could be shorter not more than about two- to three-fold (cf. Table 1) in order to obtain solid results. Nevertheless, the procedure proved to be acceptable for common laboratory purposes allowing running a series of 30 samples in 6 h with the aid of an autosampler. Additional extensive washing of the column with 80% methanol after every series proved to be useful in order to preserve unchanged retention times and peak shape.

Kinetic studies

The main role of different isoenzymes of CK in cardiac muscle is to form a 'creatine shuttle', which means that the ATP synthesized in the mitochondrial matrix is used by MtCK for the synthesis of PCr in the mitochondrial intermembrane space to form a new energy-rich phosphoryl product; PCr then enters the cytoplasm and is transported to the myofibril by a facilitated diffusion mechanism. In myofibrils the energy-rich bond is 'returned' to ADP by another, MM CK isoenzyme to form locally a newly synthesized ATP, which ensures the muscle contraction in the myosin ATPase reaction (see Fig. 4 for details).

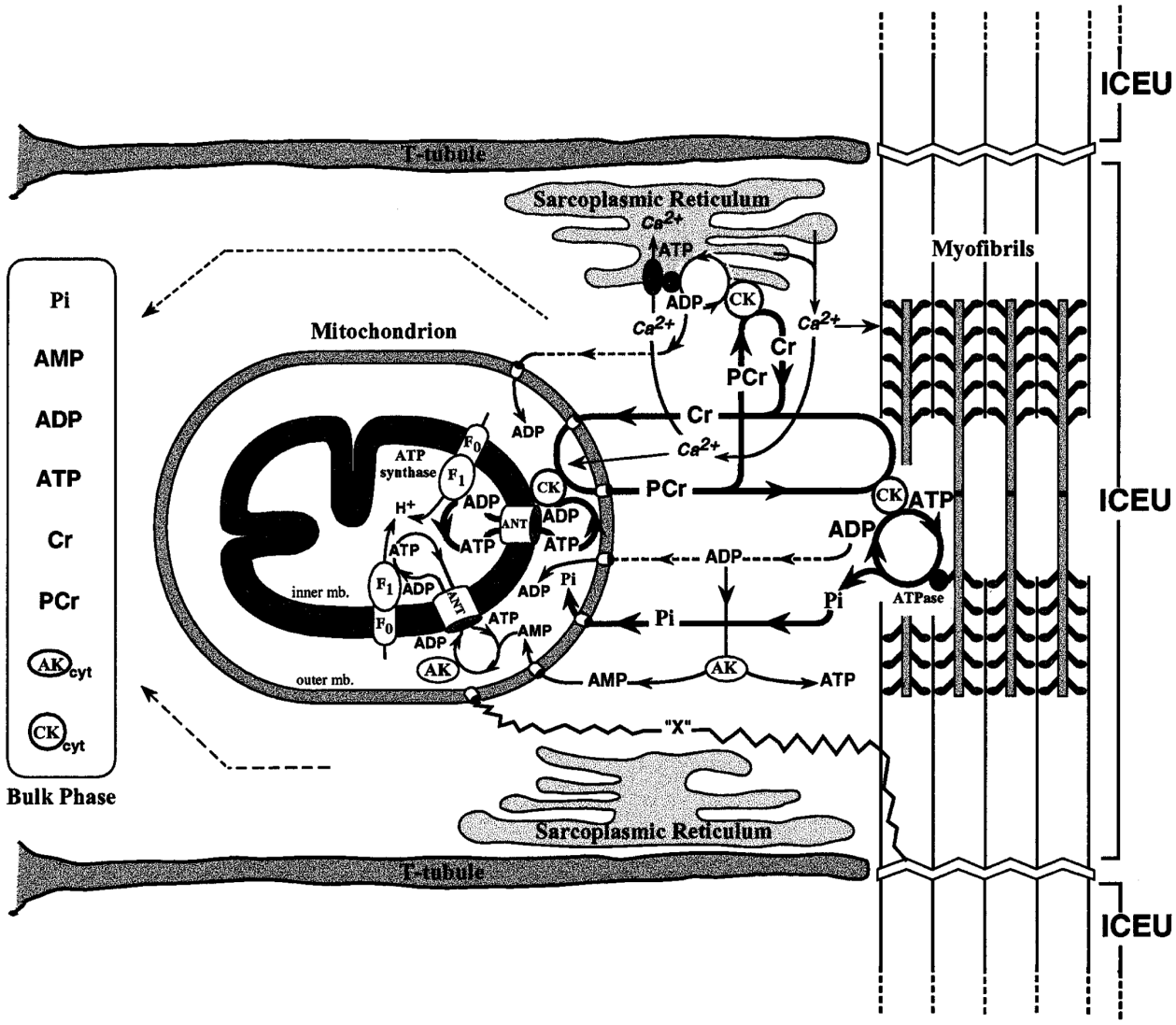

Fig. 4. Structural organization of the energy transfer networks of coupled CK and AK reactions within the intracellular energetic units (ICEUs) in cardiac cells (Saks et al., 2001, with permission). By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum (SR) are precisely fixed with respect to the structure of sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Calcium is released from SR into the space in ICEU in the vicinity of mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within ICEU do not equilibrate rapidly with adenine nucleotides in the bulk water phase. The mitochondria, SR, and MgATPase of myofibrils and ATP-sensitive systems in sarcolemma are interconnected by metabolic channelling of reaction intermediates and energy transfer within ICEU by the creatine kinase (CK)–phosphocreatine (PCr) and adenylate kinase (AK) systems. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within ICEU and bulk water phase may be connected by some more rapidly diffusing metabolites as creatine and PCr.

In relaxed cardiomyocytes supplemented by ADP and Cr one can simultaneously follow (1) the turnover of resynthesis of ATP in the matrix due to the respiratory substrates (malate and glutamate) in oxydative phosphorylation cascade reactions and (2) the transfer of the high-energy phosphoryl bond from ATP to Cr to form PCr in the CK reaction. Figure 5 shows a decrease in the ADP concentration to a low steady-state level. When plotted according to a formal (modified) first-order rate equation:

$$c = c_0 e^{-kt} + B, \qquad (2)$$

where c_0 denotes the initial $[ADP]_0 = 500$ μM, B the final steady-state ADP concentration, and k is the rate constant, the data gave the steady-state ADP concentration $B = 27 \pm 3$ μM along with the half-life time value $t_{½} = 0.63 \pm 0.17$ min in the present case (calculated from the rate constant k in Eq. 2). The corresponding increase in the ATP concentration from zero to the steady-state limit gave the value 445 ± 14 μM along with the $t_{½} = 0.75 \pm 0.04$ min, which agrees well with the above $t_{½}$ for ADP disappearance. At the same time, the synthesis of PCr from Cr and ATP shows a permanent, nearly linear increase without any visible limits. The steady-state levels of ADP and ATP reflect well-known diffusion restrictions for adenine nucleotides (see for review Vendelin et al., 2004, 2007) for passing through the mitochondrial porin channel (VDAC) as well the absence of these restrictions for PCr – which should reach the cellular cytoplasm.

In another series of experiments, the CK reaction (Eq. 1) in cardiomyocytes was initiated in the presence of an excessive amount of PK and its substrate, phosphoenolpyruvate (PEP), which resulted in irreversible conversion of ADP produced in myofibrils and other reaction sites (cf. Fig. 4) into pyruvate, thus acting as an ADP trap:

$$\mathrm{ADP + PEP \rightarrow ATP + Pyruvate.} \qquad (3)$$

In these conditions, ADP for the ATP resynthesis in the mitochondrial matrix is supplied exclusively by the reaction of MtCK isoform in the mitochondrial intermembrane space, which enables to determine the rate of this particular reaction avoiding possible contributions from other CK isoforms as well as from the hexokinase reaction (Gellerich and Saks, 1982). In these conditions, the ATP concentration remained on the steady-state level (4.60 ± 0.34 mM, cf. Fig. 6a), which corresponded

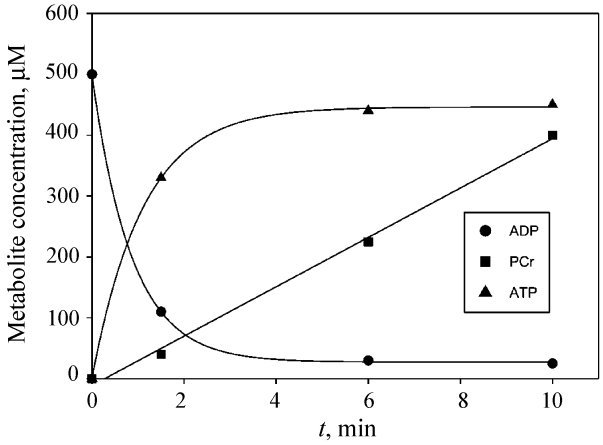

Fig. 5. Time-dependence of the phosphocreatine synthesis in permeabilized cardiomyocytes. The reaction initiated by 500 μM ADP and 10 mM creatine (n = 3–4). Note the attainment of the steady-state ADP (circles) and ATP (triangles) levels along with the gradual increase in the phosphocreatine concentration (squares).

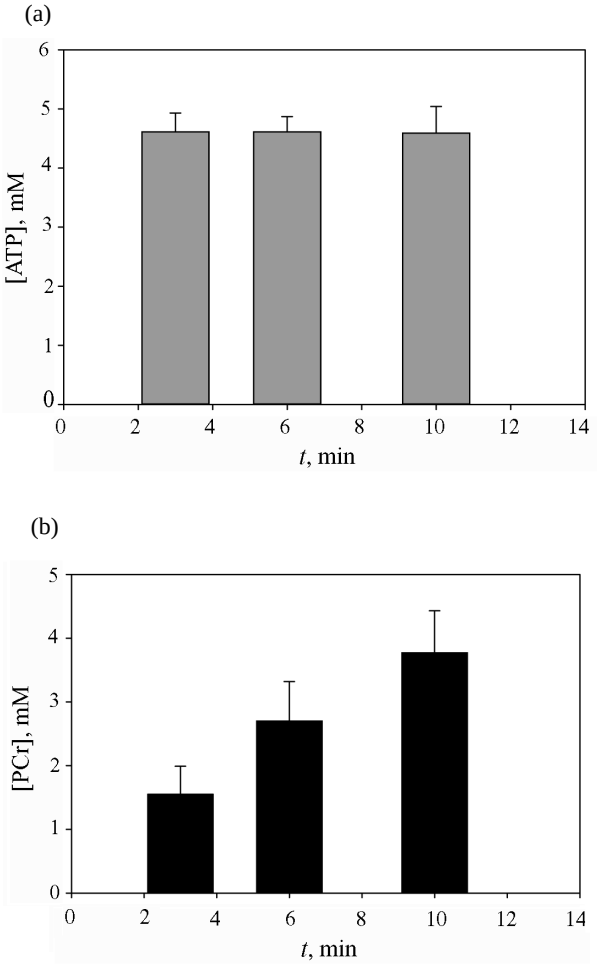

Fig. 6. Time-dependence of the phosphocreatine synthesis in permeabilized cardiomyocytes catalysed by mitochondrial creatine kinase. The reaction initiated by 4.5 mM ATP and 10 mM creatine in the presence of pyruvate kinase–phosphoenolpyruvate trap for ADP (n = 4), see text for details. Analysis was performed after 3, 6, and 10 min of the reaction. (a) Attainment of the steady-state level of ATP. (b) PCr production.

to its initial concentration 4.50 mM, along with an increase in the PCr concentration due to the MtCK reaction in the presence of a large excess of Cr (Fig. 6b).

Simultaneous determination of the rate of unrestricted PCr release from respiring cardiomyocytes along with the attained steady-state ATP and ADP levels (Fig. 5) and determination of the rate of the reaction for MtCK in overall Cr shuttle (Fig. 6b) were the main goals of this study because the data could be used as 'landmarks' in the verification of the results of permanently developing mathematical modelling of cardiomyocyte functioning (cf. Aliev and Saks, 1997; Saks et al., 2007; Vendelin et al., 2007).

CONCLUDING REMARKS

The new generation of HPLC equipment designated as UPLC provides improved facilities in resolution power, sensitivity, and, which is particularly essential for this study, enables to increase the column recycling time 2–3-fold. When using UPLC special care should be taken in the calibration of the column in a large concentration range, particularly in the 0.005–1 mM range as in the present case. It is reasonable to fit the data to both the linear and the polynomic equation $y = y_0 + ax - bx^2$. The present study showed that the two equations cannot be discriminated on the basis of R^2 values as in all cases $R^2 > 0.99$ was observed. The choice could be made only by analysis of the distribution of residuals obtained for both fitting types, thus avoiding possible large deviations of the data from the curves at low concentrations. As an alternative used in this study, the calibration could be performed in a pertinent narrow concentration range that does not exceed an order of magnitude. Application of the described UPLC procedure enabled us to provide direct evidence of the 'recycling' of ATP and ADP inside the mitochondria due to the diffusional restrictions at VDAC located in the mitochondrial outer membrane and along with unrestricted flow-out of PCr from the cells through this channel.

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Adeniinnukleotiidide ja kreatiini derivaatide analüüs ultralahutusvõimelise vedelikkromatograafia meetodil

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On kirjeldatud südamelihase energiametabolismis osalevate ühendite – kreatiini (Cr), fosfokreatiini (PCr), ADP ja ATP – üheaegse määramise meetodit, milles traditsioonilise pööratud faasi ioonpaar-vedelikkromatograafia asemel on rakendatud hiljuti arendatud ultralahutusvõimelise vedelikkromatograafia võimalusi. Kontsentratsioonide vahemikus 0,005–1 mM on standardühendite Cr, ADP ja ATP kaliibrimiskõverad kõrge usaldustasemega (kõikidel juhtudel $R^2 > 0{,}999$) kirjeldatavad polünoomiga $y = y_0 + ax - bx^2$, kuid PCr kaliibrimiskõver lihtsa lineaarse sõltuvusega on usaldustasemel $R^2 > 0{,}998$. Meetodit rakendati, uurimaks rakusisese oksüdatiivse fosforüülimise arvel toimuvat PCr sünteesi permeabiliseeritud kardiomüotsüütides. Töös määratud ADP ja ATP statsionaarseid tasemeid ning PCr sünteesi kiirust eri tingimustes kasutatakse kardiomüotsüütide funktsioneerimise kirjeldamiseks arendatud matemaatiliste mudelite kontrollimiseks.