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Creatine Kinase Reaction in Skinned Rat Psoas Muscle Fibers and Their Myofibrils

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Summary

The aim of this study was to evaluate myofibrillar creatine kinase (EC 2.7.3.2) activity on the background of the effect of substrate channeling by myosin ATPase and to compare it with creatine kinase (CK) activity of whole skinned fibers. In order to assess CK activity, skinned fibers were prepared from the rat psoas major muscles defined by light microscopy. The activity in permeabilized fibers after treatment with saponin, Triton X-100 and Ca^{2+}-free medium reached 2.80, 6.97 and 3.32 μmol ATP min^{-1} mg^{-1} protein, respectively, when a coupled enzyme assay system with external hexokinase and glucose-6-phosphate dehydrogenase was used. Transmission electron microscopy (TEM) revealed a possible interference among activities of sarcolemmal, sarcoplasmic, myofibrillar and mitochondrial CK from persisting structures. For evaluation of the myofibrillar CK itself, a pure myofibrillar fraction was prepared. Fraction purity was confirmed by TEM and by enzymatic assays for marker enzymes. Two procedures, i.e. the coupled enzyme assay and the evaluation of phosphocreatine (PCr) concentration before and after the CK reaction, were used for measurement of CK activity in this fraction. The procedures resulted in 3.2 nmol ATP min^{-1} mg^{-1} protein and 7.6 nmol PCr min^{-1} mg^{-1} protein, respectively. These alternative approaches revealed a discrepancy between the reacting portions of PCr by more than 50 %, which provides information about the size of the effect, generally described as substrate channeling.

Key words

Muscle • Skinned fibers • Myofibrils • Creatine kinase • Substrate channeling

Introduction

The molecular aspects of muscle energetics are considered currently in relation to three groups of problems: mechanisms of force generation, chemo-mechanical conversion during contraction and operation of enzyme molecules in energetic reactions. A particular role among the energetic reactions is played by the creatine kinase reaction (Lohmann 1934), which maintains the ATP concentration stable during muscular activity. The reaction is catalyzed by different isoenzymes of creatine kinase (CK) within the different

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compartments in a muscle fiber. Ubiquitous mitochondrial CK isoenzymes (Mi_a and Mi_b CK) (Jacobs *et al.* 1964, Hossle *et al.* 1988, Payne *et al.* 1991) are coupled to oxidative phosphorylation (Wyss *et al.* 1992). The cytosolic creatine-kinase isoenzymes (MM and BB CK) (Eppenberger *et al.* 1967, Pickering *et al.* 1985) in the cytosol are free. The last form is bound to subcellular structures such as the sarcolemma, sarcoplasmic reticulum or myofibrils (for review see Wallimann *et al.* 1992). It is functionally coupled to the ATPases.

The myofibrillar CK yields 5-10 % MM CK in skeletal muscles (Wallimann *et al.* 1977, 1984), depending on the muscle fiber type. It is localized mostly at the myofibrillar M-band of sarcomeres. This localization was described as being isoenzyme specific (Wallimann *et al.* 1983a). Only MM CK, and not BB CK or heterodimer MB CK, is located in this region. The MM CK molecules are functionally coupled to myosin ATPase and probably to glycolytic enzymes associated with the filaments (Wegmann *et al.* 1992).

Since the myofibrils make up functionally in a straightforward manner the macroscopic integrative behavior of a skeletal muscle fiber (Friedman and Goldman 1996), they can be considered as the simplest structure which still preserves a cellular function, and simultaneously enables access to the molecular level of energetically significant enzymes.

The present work evaluates the creatine-kinase activity in skinned muscle fibers and in isolated purified myofibrils on the background of the effect of substrate channeling between MM CK and myosin ATPase. Our activity data together with data on the structural role of CK in the myofibrillar architecture (Wallimann *et al.* 1983b, Strehler *et al.* 1983) represent a starting point for a further structural operation study of myofibrillar CK molecules integrated in the macroscopic chemical reaction.

Methods

Animals and muscle preparation

Muscle fibers and myofibrils were obtained from the psoas major muscle of Wistar male rats (350- 480 g). Under general anesthesia (50 mg kg^{-1} of sodium pentobarbital, i.p.), both psoas muscles were prepared and thereafter the rat was sacrificed. Freshly dissected intact muscle fibers were morphologically inspected by light microscopy. Transversally cut fiber bundles of about 2 mm in diameter and 2-3 cm long were kept on ice and used for the following procedures.

Skinning procedures

Single fiber segments or small bundles of several (up to ten) fiber segments were separated from fresh fiber bundles and chemically skinned using three methods: saponin, Triton X-100 and Ca^{2+}-free medium according to the modified procedure described by Thirlwell *et al.* (1994). The composition of the skinning Ca^{2+}-free medium was calculated by means of a computer program, using the equilibrium constants of Fabiato (1981). The Ca^{2+}-free medium contained (in mM): 6.34 $MgCl_2$, 76.55 KCl, 100 N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 5 sodium azide, 5 ethylene glycol-bis(β-amino-ethyl ether)-N′, N′, N′, N′-tetraacetic acid (EGTA), 5 Na_2 ATP and 10 Na_2 creatine phosphate. Muscle fibers were incubated with the medium on ice under gentle stirring for 24 h. Two other skinning solutions contained in addition Triton X-100 (1 % vol/vol) or saponin (50 μg ml^{-1}) and with these fibers were skinned on ice under gentle stirring for 3 and 1.5 h, respectively.

Enzymes activity measurements

The CK activity was directly measured using a coupled assay system consisting of hexokinase and glucose-6-phosphate dehydrogenase which resulted in the formation of NADPH (Rosalki 1967). One enzyme unit (IU) is defined as the amount of an enzyme necessary to catalyze the formation of 1 μmol ATP per min. Samples were incubated in 37.42 mM 2,2',2''-nitrilotriethanol (triethanolamine, TEA) buffer, pH 7.5 containing (in mM): 7.72 $MgCl_2$, 38.57 glucose, 0.26 Na_2 ADP, 0.57 NADP, 5.86 IU ml^{-1} hexokinase and 1.37 IU ml^{-1} glucose-6-phosphate dehydrogenase. After a 2-min incubation (in order to determine a possible contamination by myokinase activity), the reaction was started by addition of 0.1 mM creatine phosphate.

The method does not allowed an evaluation of maximum enzyme activities, nevertheless it provided sufficient data for the relative comparison of CK activities under different conditions used in this study.

Myosin ATPase activity was determined according to Arrio-Dupont (1988) in a 50 mM TEA buffer, pH 7.2, containing (in mM): 75 KCl, 10 $MgCl_2$, 0.1 EGTA, 50 μM phosphoenolpyruvate, 4 Na_2 ATP, 10 μM NADH, 1.25 IU ml^{-1} pyruvate kinase and 1.25 IU ml^{-1} lactate dehydrogenase. The reaction was started by adding a sample aliquot.

Contaminant enzyme activities of pyruvate kinase and lactate dehydrogenase were determined using standard spectrophotometric methods (Ventura-Clapier *et al.* 1995). Succinate dehydrogenase activity was

measured by the spectrophotometric method with $K_3Fe(CN)_6$ (Veeger *et al.* 1969). Lysosomal marker activity of acid phosphatase was measured by the fixed time method using *in vitro* diagnostic assay (Lachema Diagnostica).

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Preparation of myofibrillar fraction

The rat psoas major muscle, cut into small pieces immediately after killing of the animal, was transferred into a homogenization medium (in mM): 100 KCl, 1 EGTA, 5 [ethylenedinitrilo]tetraacetic acid (EDTA), 0.1 β-mercaptoethanol, 0.1 phenylmethylsulfonyl fluoride (PMSF) and 3 sodium azide (at pH 7.0) with 50 % (vol/vol) glycerol. After penetration of glycerol (about one hour), the samples were replaced into a glycerol-free homogenization medium and then homogenized according to Wallimann *et al.* (1984). The homogenates were allowed to stand on ice for 20 min and then centrifuged at 1500 *g* for 10 min. This washing procedure was repeated seven times. After the washing procedure, the myofibrillar fraction was incubated with 1% Triton X-100 (vol/vol) to eliminate the proteins loosely associated with myofibrils and other possible membrane contaminants (Solaro *et al.* 1971). After this, washing was again repeated for seven times.

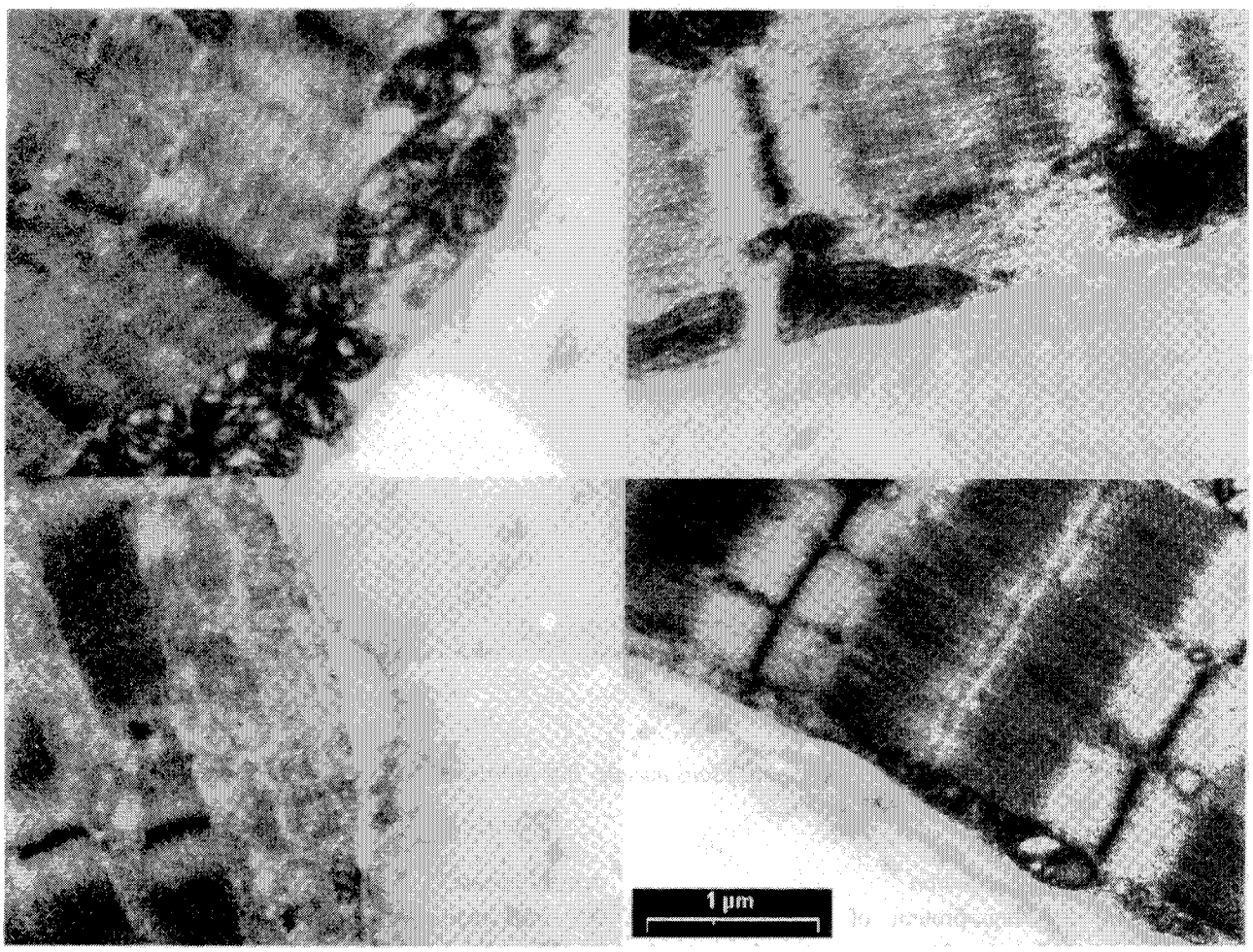

Fig. 1. *Photomicrographs of the skinned fibers prepared from rat psoas muscle. In the upper-left field is a fiber incubated in a Ca^{2+}-free medium, the upper-right field represents a fiber treated with saponin, lower-left field shows a fiber incubated in Triton and a non-skinned fiber is presented in the lower-right field. Bar represents 1 µm.*

Electron microscopy (TEM)

Extensively washed skinned fibers or the myofibrillar fraction were fixed (12 h at 4 °C) in glutaraldehyde (3 % in a cacodylate buffer, pH 7.2). After fixation, the suspension was washed twice with the cacodylate buffer and gently centrifuged. The samples were postfixed with 1 % OsO_4 at 4 °C for 3 h. The fixed material was dehydrated in ethanol and acetone series. The samples were embedded into Vestopal W resin (Fluka). Thin sections were cut on a LKB Ultratome 1 and contrasted with uranyl acetate and lead citrate according to Reynolds (1963). Electron microscopic examinations were carried out in a Philips CM100 equipped with Gatan 673 wide angle CCD camera.

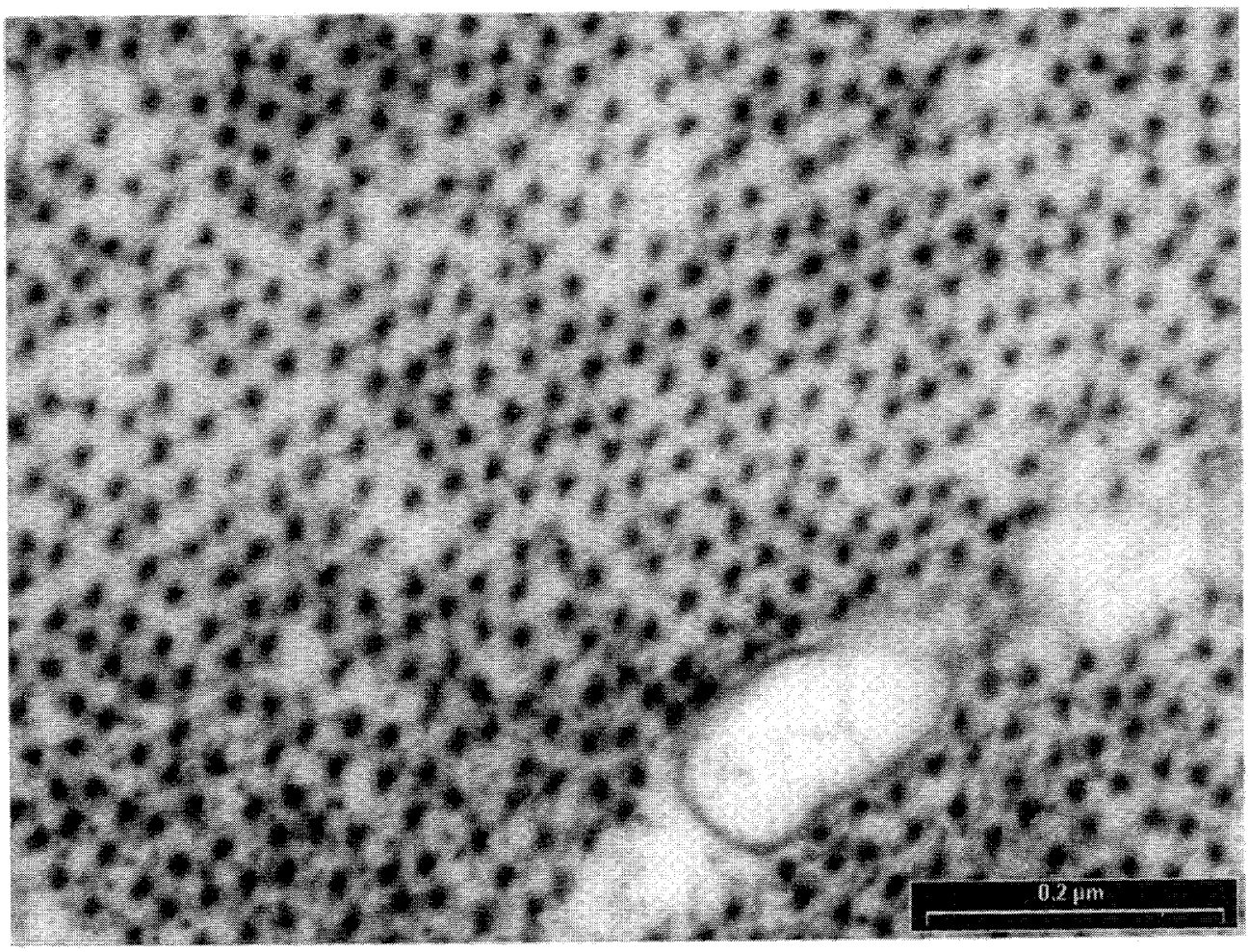

Fig. 2. *Transverse section of a fiber from the rat psoas muscle. Bar represents 0.2 µm.*

Substrate channeling evaluation

Using 2.5 mg protein of the myofibrillar fraction as a sample, the CK activity was determined during 30 min incubation in the presence of coupled enzymes. The incubation was performed in 37.42 mM TEA buffer, pH 7.5 containing (in mM): 7.72 $MgCl_2$, 38.57 glucose, 1.4 Na_2 ADP, 0.57 NADP, 5.86 IU ml^{-1} hexokinase and 1.37 IU ml^{-1} glucose-6-phosphate dehydrogenase. The reaction was started by addition of 0.53 mM creatine phosphate. Absorbance was measured continuously, using Shimadzu recording spectrophotometer UV-1601.

The amount of phosphocreatine before and after incubation was compared in parallel samples in the same solution. After incubation, sample proteins were precipitated with perchloric acid. The pH of the sample

was adjusted to 7.0 and the amount of phosphocreatine was then spectrophotometricaly determined using the coupled enzyme system.

All enzymes were obtained from Boehringer (Germany), substrates and chemicals were from Sigma (USA).

Results

Skinned fibers

Single muscle fibers observed in the light microscope take their origin from tendons on the transverse processes of lumbar vertebrae and run without interruption for a suprisingly long distance of up to 11 mm, towards their tendon insertion on the lesser trochanter of the femur. Their diameter remains the same, namely 13 μm along the whole length of the cylindrical fibers.

The skinned fibers prepared by three skinning procedures manifested very good permeability of their sarcolemma. Exogenous substrates as well as enzymes penetrated into the fibers. Direct measurements of creatine kinase activity comprising particular activities of all isoforms gave approximately the same values. The total CK activity reached 2.80, 6.97 and 3.32 μmol ATP min^{-1} mg^{-1} protein in fibers treated with saponin, Triton X-100 and the Ca^{2+}-free medium, respectively. Relatively small discrepancies among the activities confirmed that sarcolemmal permeabilization by three skinning procedures was similar.

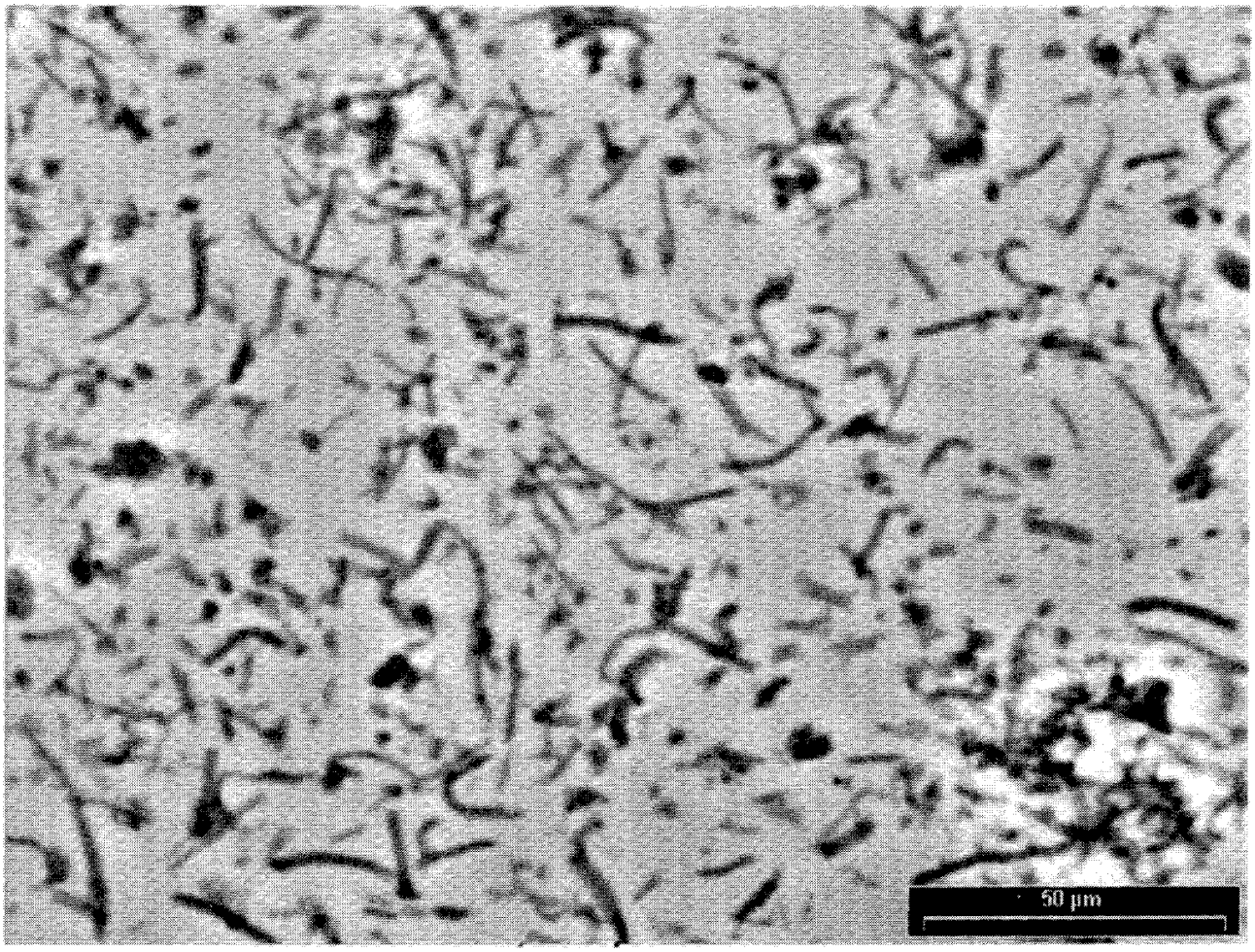

Fig. 3. *A photomicrograph (phase contrast) of the pure myofibrillar fraction prepared from the rat psoas muscle. Bar represents 50 μm.*

A TEM comparison of skinned fibers prepared by the different procedures, is shown in Figure 1. In all preparations, the skinned fibers retained their proper contractile apparatus and the samples had a typical pattern of sarcomeres which is characteristic for skeletal muscles under physiological conditions. The

mitochondrial matrix was removed in all samples, however, the Triton treatment was the most efficient. The M-line with myofibrillar CK can be recognized in treated fibers as well as in control samples. None of the skinning procedures removes the basal membrane around the muscle fibers and TEM shows remaining structures of the sarcoplasmic reticulum, mitochondria and the sarcolemma. Statistical evaluation of six electron micrographs (Fig. 2) of cross-sectioned myofibrils gives the distance between two thick myosin filaments: centre-to-centre as 31.5 nm, surface-to-surface 14.4 nm.

Myofibrillar fraction

The myofibrillar fraction represented by light microscopy contains uniform myofibrillar fragments (Fig. 3) of considerable purity, without any contaminant. The enzymes activity profile (Table 1) of the prepared myofibrillar fraction shows low or no activity of all contaminant enzyme markers. The specific activity of myofibrillar CK reached 0.003 IU mg^{-1} protein, which corresponds to 0.75% of CK activity measured in a homogenate. The CK activities during each step of preparation of the myofibrillar fraction are shown in Figure 4. In accordance with previous observations (Wallimann *et al.* 1984), direct measurement gives a stable activity value of the myofibrillar bound enzyme after five washing cycles. However, the washed CK activity was significantly lower in each further washing, the portion of the removed CK stays suprisingly constant in each washing step. This also applies to the value of the remaining bound CK after up to 10 washing cycles.

Table 1. Specific activities of CK and four contaminant enzymes (as indicators of the fraction purity) in the pure myofibrillar fraction. The percentage of enzyme activity is calculated from the total activity measured in the myofibrillar fraction and the total activity in the whole muscle homogenate. Creatine kinase activities were measured using an enzyme-coupled assay (1) and evaluation of PCr concentration (2).

	μmol min^{-1} mg^{-1} protein	%
pyruvate kinase	0.0015	0.05
lactate dehydrogenase	0.0030	0.08
acid phosphatase	–	–
succinate dehydrogenase	0.2760	9.58
creatine kinase[1]	0.0032	0.31
creatine kinase[2]	0.0076	0.75

When the myofibrillar fraction was incubated in a low ionic strength medium, most of the myofibrillar portion of CK was eluted. The removal of the bound enzyme was monitored by direct measurement of CK activity and by SDS denaturing electrophoresis. After protein collection with centricon concentrator (Amicon, cut-off 30 000), the recovery of the CK specific activity gives 0.0048 IU mg^{-1} protein corresponding to 1.2 % of the CK activity measured in a homogenate.

Further checking of the myofibrillar fraction purity using TEM (picture not shown) revealed neither contamination by other subcellular particles, nor stacked myofibrillar bundles.

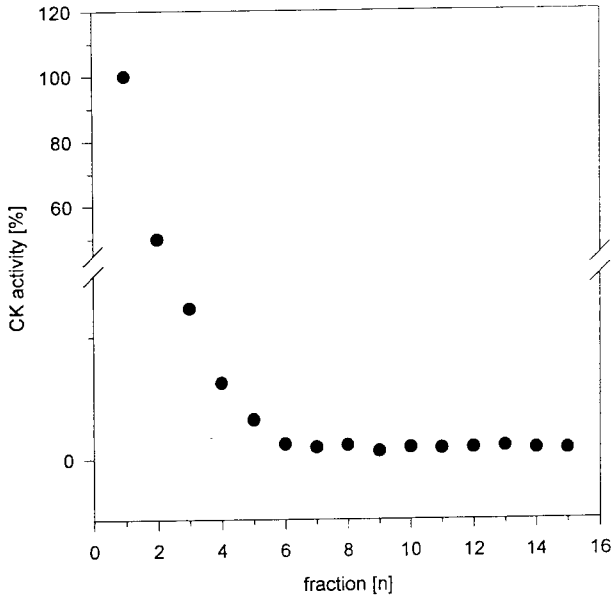

Fig. 4. *Portion of MM CK bound to the myofibrils. Creatine kinase activity is expressed as percentage of the total activity (100%, measured in the whole muscle homogenate, which represents fraction No. 1). n is the fraction number of each washing step of the preparation of pure myofibrils.*

Substrate channeling evaluation

Repeated activity measurements of myofibrillar CK were performed using 2.5 mg protein of the myofibrillar fraction as a sample after 30 min incubation in the presence of coupled enzymes (Table 1). Two procedures, i.e. the enzyme-coupled assay and the evaluation of phosphocreatine (PCr) concentration before and after CK reaction, were used to quantify substrate channeling. Changes in ATP concentration (evaluated by

the enzyme-coupled assay) were the same as those obtained in PCr concentrations for expression of CK activity. The enzyme-coupled assay allowed measurement of free accessible concentrations of ATP entering the CK reaction, i.e. reduced by ATPase activity of myosin. Measurements of PCr concentration before and after CK reaction avoid the competitive effect of myosin ATPase. Thus, a comparison of the two procedures could be used for quantification of substrate channeling at the level of myofibrillar CK. The former procedure gave results of 3.2 nmol min^{-1} mg^{-1} protein, while the latter gave values of 7.6 nmol min^{-1} mg^{-1} protein of reacted ATP and PCr, respectively. These alternative approaches revealed a discrepancy between reacting portions of PCr of more than 50 % (4.4 nmol min^{-1} mg^{-1} protein), indicating the extent of substrate channeling of the CK reaction by myofibrillar ATPase *via* ATP.

Discussion

Skinned fibers are considered to be an ideal model for studies dealing with muscle physiology at the cellular level under persisting native conditions. This experimental model has been successfully used for studies in the field of muscle contraction (Hilber and Galler 1997, Coonan and Lamb 1998, Veigel *et al.* 1998), signal transduction (Kuznetsov *et al.* 1996, Galler *et al.* 1997) and energy metabolism (Potma and Stienen 1996, O'Gorman *et al.* 1997, Sahlin *et al.* 1998). Nevertheless, skinned fibers have some structural and biochemical disadvantages for further studies of the behaviour of myofibrillar CK molecules in a macroscopic energy reaction. These disadvantages make it necessary to simplify muscle fibers to a purely myofibrillar fraction.

The structural disadvantage of whole muscle fibers consists in the restricted accessibility of enzyme molecules in fiber bundles to external substrates or testing enzymes and by their restricted diffusion within a fiber through the actomyosin molecular sieve (Arrio-Dupont *et al.* 1997, Tanner *et al.* 1992). In spite of this fact, CK activity measured in the skinned fibers is found to be higher as it is measured for myofibrillar CK in the pure myofibrillar fraction from the whole muscle homogenate. The unexpected difference is due to the high content of non-myofibrillar CK isoenzymes.

Possible interference of Mi-CK activity (5-20 % of the cellular CK, Wegmann *et al.* 1992), due to persisting mitochondria observed by TEM microscopy, is one of the biochemical obstacles for evaluation of pure myofibrillar CK activity in the energetically active skinned fibers.

However, contrary to the skinned fibers, pure myofibrillar fraction exhibits ideal biochemical homogeneity and well defined relations between structure and function. Further washing procedures together with Triton treatment significantly reduced the content of contaminating enzymes. Fraction purity, documented by marker enzyme activities (Table 1) was achieved at the expense of a relatively low yield of myofibrillar CK. The previously described relative portion of the myofibrillar CK activity, with respect to the total CK activity, represented 5 to 10 % (Wallimann *et al.* 1977, 1984). In our preparations, 5 % activity values (see Fig. 4), considered as the total CK localized in myofibrils, was repeatedly obtained during 4-5 wash cycles. The portions of myofibrillar CK removed between washing cycles 5 and 10 correspond to the activity of loosely bound CK localized mostly to the I-band of the sarcomere (Wegmann *et al.* 1991, 1992). The remaining CK activity measured after 10 washing cycles represented unremovable, tightly bound myofibrillar CK localized to the M-band (Wallimann *et al.* 1983b, Stolz and Wallimann 1998).

Substrate channeling represents another biochemical obstacle which restricts the determination of absolute activity of the myofibrillar CK isoenzyme. Even in the pure myofibrillar fraction, this effect has been observed. Arrio-Dupont (1988) first described substrate channeling of myofibrillar CK in frog ventricular cells. This problem was noted in several papers (e.g. Saks *et al.* 1994, 1996), without being quantitatively evaluated. Direct measurement of total PCr consumed in the CK reaction, gives a reasonable estimation of the channeling effect in our experiments. The extent of 60 % ATP channeling is in good correlation with the results obtained by comparison of CK activity determined in the pure myofibrillar fraction and after elution in a low ionic strength medium. Due to elution of all myofibrillar CK (92-96 %, Wallimann *et al.* 1984), the portion of myofibrillar CK activity rose to twofold values. The biochemical role of substrate channeling has been extensively discussed elsewhere (Cornish-Bowden and Cárdenas 1992, Ovádi 1995, Kholodenko *et al.* 1996).

Acknowledgments

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