*Biochimica et Biophysica Acta*, 974 (1989) 74–80
Elsevier

BBA 42971

# Ultrastructural localisation of creatine kinase activity in the contact sites between inner and outer mitochondrial membranes of rat myocardium

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(Received 13 October 1988)

Key words: Creatine kinase; Contact site; Mitochondrial membrane; Myocardium; Electron microscopy; (Rat)

**The mitochondrial isoenzyme of creatine kinase, together with the ADP/ATP translocase, most probably belongs to a functional multi-enzyme complex located on the inner mitochondrial membrane. The outer membrane is a necessary constituent of this microcompartment. On the other hand, electron microscopic visualisation demonstrated the formation of contact sites between inner and outer mitochondrial membranes as a reaction to variations of the energy metabolism. In search for a possible correlation between these biochemical and morphological phenomena, rat myocardia were brought into the required energy state by stimulation through catecholaminergic mechanisms or adjusted perfusion with amytal. Subsequently, creatine kinase was cytochemically localised. Creatine kinase activity is demonstrated in membrane contacts between inner and outer mitochondrial membranes. The extent of contact sites and creatine kinase activity depends on the metabolic state as shown by morphometric analysis of the surface density of cytochemical reaction product. This surface density diminishes drastically after inhibiting the metabolic activity with amytal. It is concluded that these contact sites are dynamic micro-environments in which the active site of creatine kinase, oxidative phosphorylation and ADP/ATP transport interact during basal and stimulated metabolism.**

## Introduction

The enzyme ATP: creatine phosphotransferase (EC 2.7.3.2) catalyses the reversible transfer of a phosphate residue between ATP and creatine. The reaction product, creatine phosphate, is a vehicle for transport of energy within tissues and cells of high-energy demand, e.g., brain, muscle, retina and spermatozoa [1]. Creatine kinase (CK) therefore is to be considered as a key enzyme of muscle metabolism, especially myocardium. In this tissue, various CK isoenzymes, mitochondrial CK MiMi, sarcoplasmic CK MM, myofibrillar bound CK MM, participate in the so-called creatine phosphate shuttle, a process that cares for the intracellular energy transport [2–4]. In heart muscle, 30% of CK activity is performed by the mitochondrial isoenzyme [5] located on the cytoplasmic side of the inner membrane [6–8]. It has been reported that active mitochondrial CK belongs to a microcompartment where ATP, produced by oxidative phosphorylation, is preferentially used by the phosphotransferase, whereas ADP is transported back to the mitochondrial matrix via the ADP/ATP translocase, thus stimulating respiration [9–15]. The outer mitochondrial membrane seems to be a necessary functional constituent of this compartment [11,16].

On the other hand, ultrastructural changes following variations of the energy metabolism have been demonstrated in heart mitochondria, both in vivo and in vitro. Indeed, it appears from electron microscopic observations of thin tissue sections and freeze-fracture preparations of isolated mitochondria, that the frequency of contact and semi-fusion sites between inner and outer membrane is changing according to the energy state of the mitochondrion [17–20]. For instance, strong catecholaminergic stimulation decreases the mean distance between inner and outer membrane and increases the number of contact sites. After partial inhibition of the respiratory chain by amytal, the mean intermembrane distance increases and the number of contact sites diminished substantially [17,18].

The aim of the present investigation was to search for a possible link between these biochemical and morphological phenomena; in other words, the correlation

Abbreviation: CK, creatine kinase.

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between the energy state of the myocardium and the ultrastructure of the rat heart mitochondrion, especially the appearance of contact sites and the location of CK activity.

## Materials and Methods

Ten female Wistar rats (200–250 g body weight) were anaesthetised by intraperitoneal injection with Nembutal® (80 mg/kg body weight) and divided into two groups, each of five animals, which received different treatments. The hearts of the first group of animals were retrogradely perfused via the aorta dorsalis with ice-cold Tyrode's medium (137 mM NaCl/2.7 mM KCl/1.36 mM $CaCl_2$/0.49 mM $MgCl_2$/0.36 mM $NaH_2PO_4$/11.9 mM $NaHCO_3$/5 mM glucose (pH 7.4)). The animals of the second group were first intravenously injected via the vena femoralis with amytal (30 mg/kg), after which the myocardia were perfused with Tyrode's medium containing 1.8 mM amytal. CK activity was cytochemically localised according to the slightly modified method of Farrell et al. [21].

Fixation in situ was obtained by a 10 min perfusion with 2% formaldehyde/6% glucose/6% dextran/10 mM cysteine in 50 mM cacodylate buffer (pH 7.2), followed by a 10 min perfusion with 6% glucose, buffered with 50 mM cacodylate (pH 7.2).

Tissue blocks were rinsed for 30 min in the same buffer, exposed during 30 min to 7% (v/v) dimethylsulphoxide and cut into 30 $\mu$m thick, frozen sections at $-30\,^{\circ}$C. These cryosections were incubated in the phenazine methosulphate/ thiocarbamylnitrobluetetrazolium reaction medium.

The complete incubation medium contained 1 mM ADP/15 mM AMP/330 mM glucose/1 mM $NADP^+$/6.4 mM creatine phosphate/10 mM magnesium acetate/30 U/ml hexokinase/0.15 U/ml glucose-6-phosphate dehydrogenase/1.3 mM phenazine methosulphate/0.6 mM thiocarbamylnitrobluetetrazolium salt in 50 mM Tris buffer (pH 7.2). All enzymes, nucleotides, phenazine methosulphate and thiocarbamylnitrobluetetrazolium salt were purchased from Sigma, St. Louis, MO U.S.A., and dextran ($M_r$ 60 000–90 000) from Serva, Heidelberg, F.R.G. All other reagents used were of the highest purity grade commercially available.

Cryosections were pre-incubated for 20 min in medium without creatine phosphate and $NADP^+$, followed by incubation for 60 min in complete reaction medium. Sections for cytochemical controls were incubated in media with the following adaptations: (1) after prolonged fixation in hot 2.5% glutaraldehyde solution buffered with 100 mM cacodylate (pH 7.4), (2) after inhibition with 2 mM 1-fluoro-2,4-dinitrobenzene, a specific inhibitor for CK, (3) without the essential cytochemical reagents creatine phosphate or hexokinase.

All incubations were performed in the dark and at room temperature. After incubation, sections were briefly rinsed in cacodylate buffer (pH 7.2), postfixed for 1 h at 4 °C in 1% osmium tetroxide, buffered with 100 mM cacodylate (pH 7.4) and further processed according to standard electron microscopic techniques. Ultrathin sections were cut on a LKB Ultrotome III microtome and observed unstained with a JEOL 1200 EX electron microscope at 80 kV.

The surface density of the cytochemical reaction product was determined using a ZEISS EM 902 electron microscope operated at 200 eV electron energy loss. In this condition, high material densities are visualised. Since CK activity provides a high material density to the contact sites, they are suitably discriminated from the other cell structures to be analysed with a Kontron SEM-IPS image analysis system, using the morphometric methods described [22,23].

According to Weibel [22], the surface density can be defined as the surface area of the component per unit containing volume. The surface density is directly related to the number of intersections formed with testlines of a grid lattice superimposed on the image. To minimise the effects of the anisotropic ultrastructure of heart muscle, the method as described by Baddeley et al. [23] was used.

The surface density was estimated using a coherent test system consisting of cycloidal curves and points, which was superimposed on the image. According to Baddeley et al. [23], surface density can be obtained from the equation

$$S_v = 2(p/l)\frac{\sum_{i=1}^{n} I_i}{\sum_{i=1}^{n} P_i}$$

where $p/l$ is the ratio of total number of test points to

TABLE I

*Effect of stimulation and inhibition of energy metabolism on the surface density of CK-active contact sites per mitochondrial volume*

Ten rats were used, divided in two groups, each of five animals. Two tissue blocks per myocardium were cut in sections, which were cytochemically processed. Images of one section/myocardium were taken at fifteen arbitrary chosen places (five places/maze on the grid) at a primary magnification of 12000×. Surface density of CK activity precipitate per mitochondrial volume was measured as described in Materials and Methods. Data represent mean ± S.E. for a total of 75 measurements.

| | $S_v$(mit) ($cm^{-1}$) |
|---|---|
| Stimulated | 0.68 ± 0.02 |
| Inhibited | 0.33 ± 0.01 [a] |

[a] Value is significantly different from stimulated group-operated values for $P$ values of 0.005 (Student's $t$-test).

total test-curve length, $n$ the number of images, $I_i$ the intersection counts of cytochemical reaction product with the test lines and $P_i$ the points counts falling on mitochondrial profiles.

## Results

In the first group of animals, the energy metabolism may be considered as weakly stimulated due to manipulation before anaesthesia. Numerous contacts between inner and outer mitochondrial membranes were visible in the heart muscle. An electrondense, osmiophylic formazan reaction product of CK activity was cytochemically demonstrated on the contact sites (Fig. 1 and 2A). In all cytochemical controls, i.e., sections of myocardia from the same animals incubated in media, without hexokinase or creatine phosphate, respectively, or after

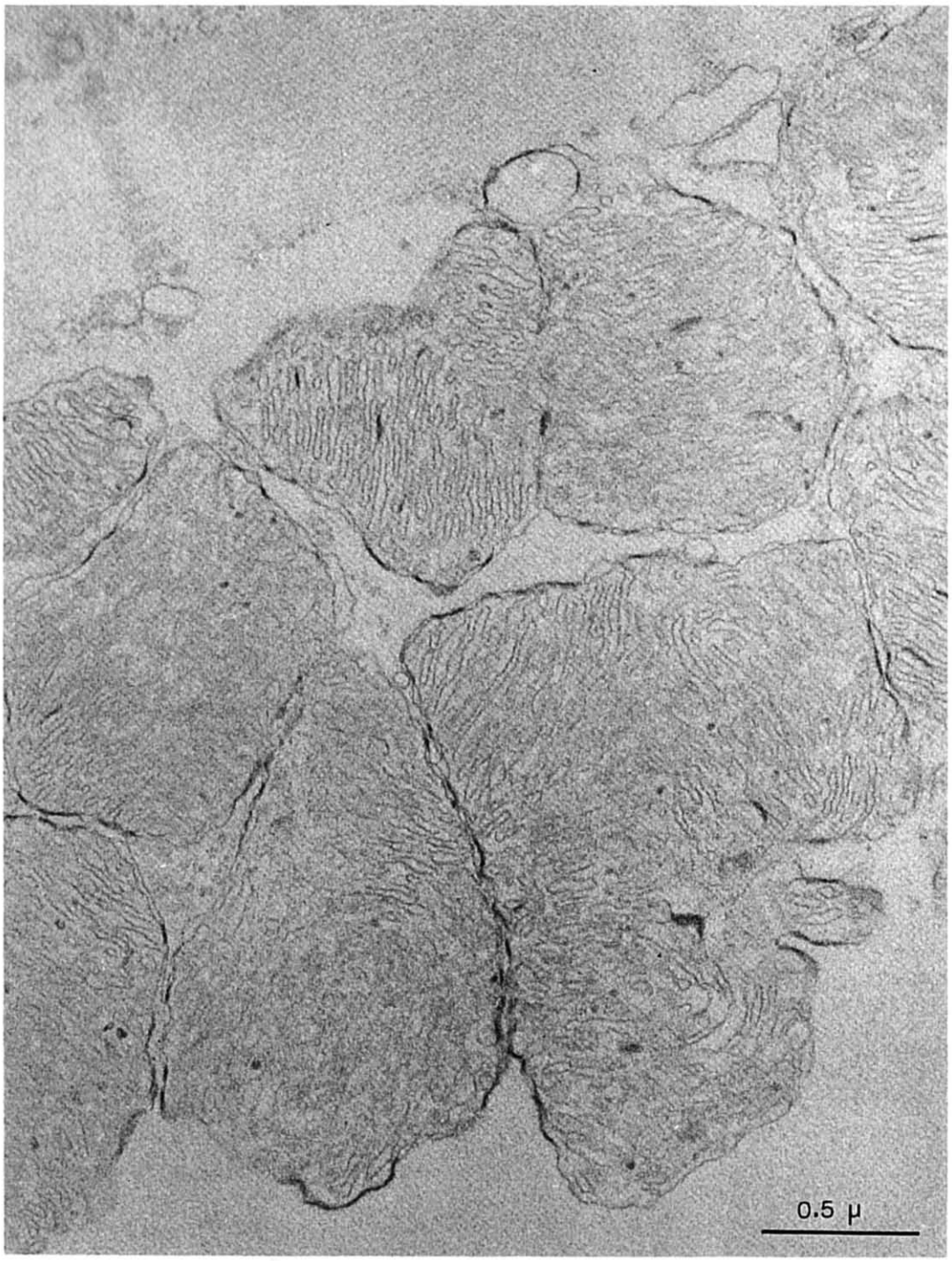


Fig. 1. Electron micrograph showing the ultrastructural localisation of CK activity in rat myocardium of a stimulated animal. CK activity is observed in contacts between inner and outer mitochondrial membranes and occasionally in the intercrystal space, where membranes are in close apposition.

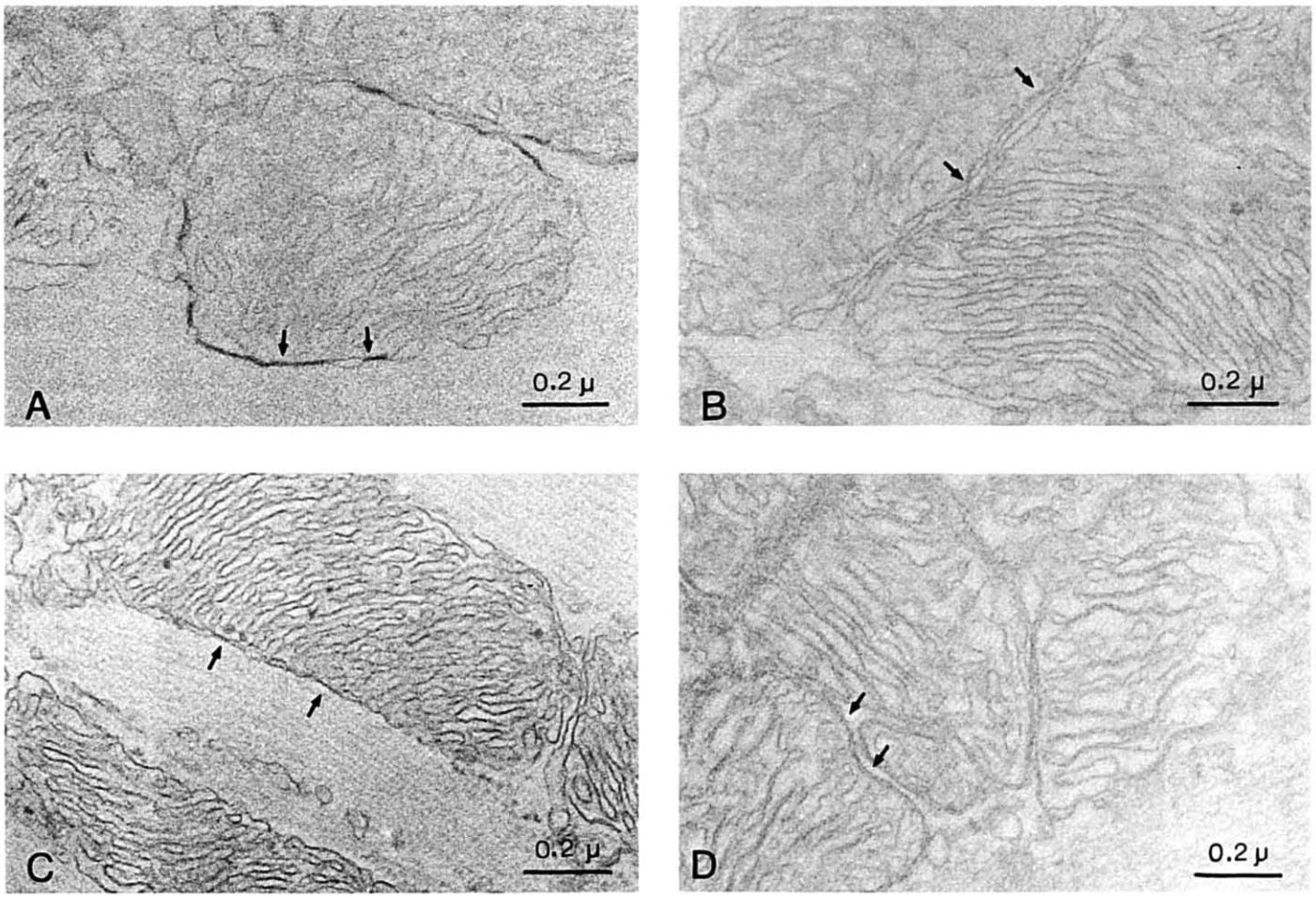


Fig. 2. Electron micrographs showing the ultrastructure of mitochondria in sections of myocardium of a stimulated animal after incubation in complete and control media. (A) Mitochondria in a section incubated in complete cytochemical medium. CK activity is localised in contact sites between inner and outer membranes (arrows). (B) Mitochondria in a section incubated in medium without hexokinase. Contact sites are visualised but without CK activity (arrows). (C) Mitochondria in a section incubated in medium without creatine phosphate. Membrane contacts are visible, but without CK activity (arrows). (D) Mitochondria in a section incubated in complete medium with addition of the inhibitor 1-fluoro-2,4-dinitrobenzene. Membranes are in close apposition but without CK activity (arrows).

addition of 1-fluoro-2,4-dinitrobenzene, membrane contacts were visible, but without detectable precipitate (Fig. 2B, C and D).

Amytal was used as an inhibitor of the respiratory chain. After this treatment, the number of membrane contacts was reduced, but CK activity persisted in association with the contact sites (Fig. 3). In this case, also, cytochemical controls gave negative results. Results of the morphometric analysis of the two groups of myocardia are shown in Table I. In this study no other cellular location showed CK activity, except occasionally in the intercrystal space, where close apposition or fusion of the crystal membranes was observed.

## Discussion

It is seen that upon energisation of the myocardial mitochondria, due to catecholamine release provoked by the manipulation of the animals, the surface density of contact sites between inner and outer mitochondrial membranes is high (Fig. 1 and Table I). The existence of these contacts has first been demonstrated by Hackenbrock in thin sections [24]. Recently, they were demonstrated in freeze-etch and freeze-fracture preparations of mitochondria [17–20,25]. Although a protein transport function for these contact sites in *Neurospora crassa* mitochondria has been proposed [26], results of other investigations point also to a possible role in the energy metabolism of heart [17,18] liver [20] and skeletal muscle mitochondria [27]. These observations are confirmed in our study. Indeed, injection of an inhibitor of the mitochondrial respiratory chain, such as amytal, leads to a decrease in oxygen consumption and phosphorylation potential [28]. However, amytal only partially inhibits the electron transport system. Energy production remains possible via complex II and III of the respiratory chain. This reduced energy production is correlated with a smaller number of membrane contacts with CK activity (Fig. 3 and Table I).

Various data from recent research indicate a possible

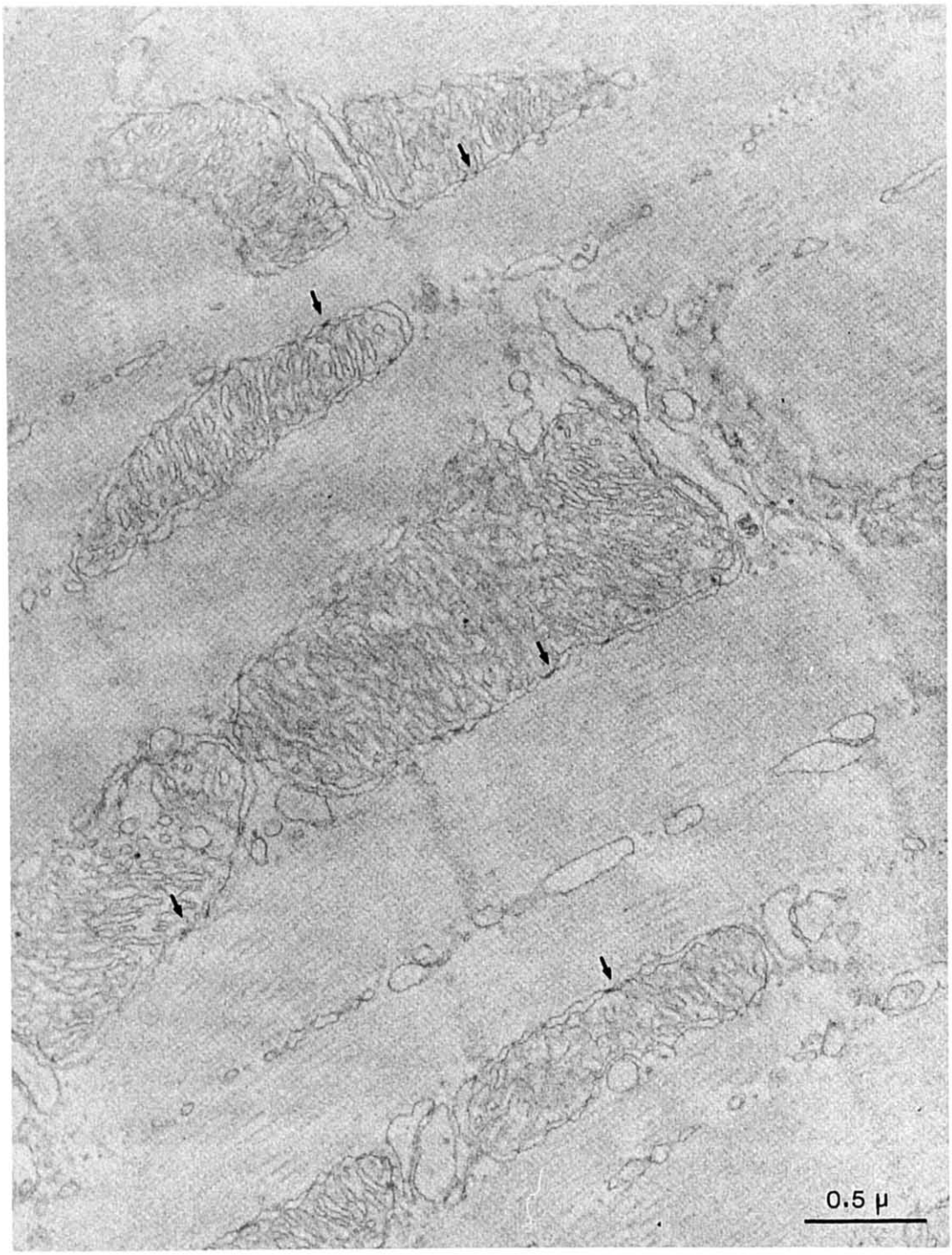


Fig. 3. Electron micrograph showing the ultrastructural localisation of CK activity in rat myocardium after inhibition with amytal. In the remaining contact sites, CK activity is demonstrated (arrows).

relation between membrane contacts and different kinases, e.g., hexokinase, nucleoside diphosphate kinase and CK in mitochondria [29–31]. In different tissues the compartmentation of kinases in mitochondria has been studied. Attention has been paid especially to the specific role of CK from heart mitochondria [15,16,32] and brain mitochondria [30]. In brain mitochondria, maximal CK activity was found to be associated with an isolated contact-site fraction [30]. In the heart muscle CK is an outstanding component of the creatine phosphate shuttle between mitochondria and myofibrils, where a specific CK isoenzyme allows creatine phosphate to regenerate ATP near the active site of the myofibrillar ATPase [3,4]. Therefore it may be surprising that we do not detect the myofibrillar localisation of CK with our method, which per se cannot discriminate between different CK isoenzymes. The very weakly bound character of the enzyme might explain this [33,34].

Indeed, washing away during the preparative steps in our method, e.g., very weak fixation and permeabilisation of membranes through dimethylsulphoxide treatment and freeze-thawing, is not unlikely. This explanation could also hold for the absence of cytochemical precipitate in the intermembrane space outside the contact sites. Nevertheless, other explanations are also possible in this case. Either the mitochondrial CK is exclusively present in the contact sites, as was shown for CK in brain mitochondria [30], or CK is present randomly on the inner mitochondrial membrane, but shows only enzyme activity in the membrane contacts. Recently, Wallimann's group [27], using immunogold labeling of retinal mitochondria with anti-CK MiMi antibodies, have shown a random distribution of gold particles following the inner mitochondrial membrane. The discrepancy in localization of CK between both cytochemical and immunocytochemical methods may be only apparent. First, in the immunocytochemical method, clustered labeling was also observed at peripheral regions where inner and outer membrane are in close apposition. Secondly, our cytochemical method only visualises CK activity and does not exclude a random distribution of inactive enzyme proteins. Wallimann's observation that the interconversion between the inactive dimeric forms and the active CK octamer could be stabilized by association with the outer mitochondrial membrane [27] may in fact be a confirmation of our cytochemical results.

In any case, data from recent biochemical studies indicate the necessity for a functional compartment [15,16,32,35]. Brooks et al. [16] assume a compartmentalized coupling between CK and the ADP/ATP translocase influenced or controlled by the outer membrane. This structure would function as a diffusion barrier to maintain high concentrations of ADP and ATP in the intermembrane space. Saks et al. [15] have put forward a directed interaction (of unknown nature) between CK and the ADP/ATP translocase by frequent collisions in a cardiolipin domain of the inner membrane. Müller et al. [32], by investigating the binding of solubilised CK MiMi to cardiolipin-containing liposomes in the presence of adriamycin, question this coupling and only confirm the binding of CK to cardiolipin. This explanation has also been suggested by Schlame et al. [35], using re-association experiments between CK MiMi and mitoplasts. The results of our study do not allow us to take side with one of the above alternatives. This study, however, does show that, since CK activity is also present in contact sites between crystal membranes, the mere fusion of mitochondrial membranes seems to be essential. It seems that this physicochemical entity is a prerequisite for CK to demonstrate enzyme activity.

In summary, the results of this study clearly show that the fundamental role which has been proposed for the contact sites in the metabolism of liver mitochondria [20] seems to hold also for myocardial mitochondria. While some studies describe the outer mitochondrial membrane as a diffusion barrier for ATP and ADP, necessary for CK activity [11,16], our data demonstrate that its importance goes beyond this. Indeed by its fusion with the inner membrane, the outer membrane creates a dynamic multi-enzyme domain for efficient production and transport of energy during basal and stimulated metabolism.

## Acknowledgements

The authors thank F. Lakiere for the preparation of the electron micrographs. This study was supported by Grant 87/92-120 of the Interministerial Commission of Science Policy, Belgium.

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