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Ultrastructural localisation of carnitine acetyltransferase activity in mitochondria of rat myocardium

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Abstract

The acetyl CoA/CoA ratio is an important regulating factor of β-oxidation in mitochondria and hence of energy production in the myocardium. Carnitine acetyltransferase provides one of the control mechanisms for this ratio during changing energy demand in the heart muscle, possibly by buffering the CoA and carnitine concentration for sustained β-oxidation. In search for a possible correlation between the activity of this enzyme and ultrastructural changes in heart mitochondria, carnitine acetyltransferase was cytochemically localised in rat myocardium, brought into different metabolic states. In this work we confirm previous observations, namely the formation of contact sites between inner and outer mitochondrial membranes upon catecholaminergic stimulation of the myocardium. It is further shown that this contact site formation might be a prerequisite for carnitine acetyltransferase to demonstrate enzymatic activity and hence control of β-oxidation in myocardial mitochondria.

Key words: Carnitine acetyltransferase; Myocardium; Mitochondrion; Contact site; Cytochemistry

1. Introduction

The enzyme carnitine acetyltransferase (CAT) (EC 2.3.1.7) catalyses the reversible reaction:

acetylcarnitine + CoA $\rightleftharpoons$ acetyl CoA + carnitine

with the equilibrium constant (K_{eq}) equal to 0.6–0.7 [1]. The pH optimum of the CAT reaction was found between 7.5 and 8.0, when measured with L-carnitine and acetyl CoA as substrates [2,3].

CAT is widely spread in animal cells and tissues with large oxidative capacity, especially in myocardium, spermatozoa and brown adipose tissue [4]. The subcellular localisation of CAT activity was found to be 78% in the mitochondria associated with the inner membrane [5] and 19% in the cytosol [2]. Although precise knowledge about the function of CAT is still not fully understood, Pearson and Tubbs [6] suggest that CAT plays a fundamental role in buffering against rapid changes in the tissue acetyl CoA/CoA ratio.

The acetyl CoA/CoA ratio is an important regulating factor of β-oxidation in the heart, as the overall rate of myocardial fatty acid utilisation is determined by the availability of exogenous fatty acids and the rate of acetyl CoA oxidation by the citric acid cycle [7].

As biochemically shown by Idell-Wenger et al. [8] a rapid communication between mitochondrial and cytosolic CAT exists, provided by the cooperating system of transferase and translocase of carnitine CoA. This creates a tight coupling between the mitochondrial and the cytosolic metabolism which might be of crucial importance in situations of high energy demand, as for example after catecholaminergic stimulation.

As demonstrated in previous work, upon stimulation important ultrastructural changes occur, namely the formation of mitochondrial contact sites functioning as a microcompartment for efficient enzyme activity and transport of metabolites [9]. These contact sites are dynamic structures created by the fusion of the inner and outer mitochondrial membranes [10].

The aim of the present study is to find a correlation between the energy state of the myocardium and the morphological phenomena, by showing that carnitine acetyl transferase needs contact sites for its activity. For that purpose, the energy metabolism of rat heart

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muscle was brought into well defined states by adjusted perfusion, after which CAT activity was cytochemically localised.

2. Materials and methods

Twenty-four female Wistar rats (200–250 g body weight) were anaesthetised by intraperitoneal injection of Nembutal® (80 mg/kg body weight) and divided into 3 groups, each of 8 animals, which received different treatments.

The hearts of the first group of animals were retrogradely perfused in situ with cold Tyrode's medium (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM Na_2HPO_4, 11.9 mM $NaHCO_3$, 5 mM glucose (pH 7.4)).

A few minutes after injection with amytal (30 mg/kg body weight) via the vena femoralis, the second group was perfused with cold Tyrode's medium containing 1.8 mM amytal.

The third group was injected with noradrenaline (0.02 mg/kg body weight) via the vena femoralis, followed by a perfusion with cold Tyrode's medium containing 0.1 mM noradrenaline.

Amytal and noradrenaline were chosen to induce two significantly different metabolic states. Amytal is known to provoke a decrease in energy demand while noradrenaline is stimulating the energy metabolism.

Biochemical characterisation

To characterise the biochemical parameters of the different situations three hearts per group were analysed. Immediately after cold cardioplegic arrest of the heart, biopsies were taken from the left ventricle and cooled with liquid nitrogen. These biopsies were lyophilised at -100°C and $1.3 \cdot 10^{-5}$ Pa in a Balzer freeze fracture device with an adjusted holder. After 1 h the temperature was increased to -80°C, followed by a gradual overnight increase to room temperature.

For analysis, the dry samples were homogenised in ice cold 0.6 N $HClO_4$ (1 ml/2 mg dry tissue) during 10 s using a polytron at 14000 rpm. After a short centrifugation, 300 μl of the supernatant was neutralised with 200 μl of cold 1 N $KHCO_3$. After centrifugation, the extract was used for further analysis.

Nucleotides, nucleosides, the bases and nicotinamide adenine nucleotide were separated by reversed phase HPLC (C_{18} column) as described by Wynants et al. [11].

Cytochemical localisation of the CAT activity

To cytochemically localise the CAT activity, Higgins's method [12] was slightly modified. Acetyl CoA and carnitine were purchased from Sigma, St. Louis, MO, USA. All other reagents used were highest purity grade commercially available.

Fixation in situ was obtained by a 15-min perfusion via the aorta dorsalis with 1% formaldehyde, 0.5% glutaraldehyde, 4.5% glucose buffered with 50 mM cacodylate (pH 7.4), followed by a 10-min perfusion with 6% glucose in 50 mM cacodylate (pH 7.4). The outflow was performed by piercing the right auricle. The tissue blocks were rinsed for 30 min in 6% glucose, buffered with 50 mM cacodylate (pH 7.4). After a 30-min immersion in 7% DMSO solution, the tissue was frozen in dry ice and 40 μm frozen sections were made at -30°C. After a short wash in 0.02 M veronal acetate (pH 7.4) with 4.5% glucose, these sections were preincubated for 45 min in 12 mM $K_3Fe(CN)_6$ in 0.02 M veronal acetate (pH 7.0) with 4.5% glucose. The change to veronal acetate was done to avoid precipitation of uranylacetate in the incubation medium. Between the pre-incubation and the incubation for 45 min in a medium containing 6 mM $K_3Fe(CN)_6$, 2.35 mM uranylacetate, 0.91 mM acetyl CoA, 8.1 mM L-carnitine in 0.02 M veronal acetate (pH 7.0) with 4.5% glucose, the sections were repeatedly washed for 30 min. After the incubation at room temperature, the sections were rinsed repeatedly during 30 min with 4.5% glucose in 0.02 M veronal acetate (pH 7.4), postfixed for 1 h at 4°C in 1% osmium tetroxide, buffered with 0.02 M veronal acetate (pH 7.4) and further processed according to the standard electron microscopical techniques. As cytochemical controls, the sections were incubated in media without the essential cytochemical reagents carnitine and acetyl CoA.

Ultrathin sections were cut on an LKB Ultratome III microtome and studied with a JEOL 1200EX electron microscope at 80 kV.

Morphometry

To get a more stringent, quantitative evaluation, we performed morphometric analysis on the surface density of the cytochemical reaction product.

The myocardium from each animal in each group was divided into four pieces from which two were chosen and prepared for cryosectioning. The cryosections prepared from these two samples were pooled and cytochemically processed. A minimum of six cryosections thus prepared were embedded and two blocks were randomly chosen and sectioned. On these grids, an average of twenty fields were photographed systematically at a magnification of 15000. About 100 pictures per group were taken.

The surface density of the cytochemical reaction product in the mitochondrial contact sites was determined using the system of Baddeley et al. [13]. To minimise the effect of the anisotropic ultrastructure of heart muscle, Baddeley described a coherent test system consisting of cycloidal curves superimposed on the image. The surface density is directly related to the number of intersections formed with the test lines of

the lattice. The surface density can be obtained from the equation

$$S_S = \frac{\sum_{i=1}^{n} I_i}{\sum_{i=1}^{n} S_i}$$

where I_i represents the intersection counts of cytochemical reaction points with the cycloidal test lines, and S_i is the total amount of intersection counts of the mitochondrial membrane with the cycloidal test lines.

3. Results

Biochemical data

To characterize the different treatments biochemically, we have calculated the energy charge based on HPLC analysis of ATP, ADP and AMP (Table 1). The energy charge (EC) is calculated as:

$$EC = \frac{2\,ATP + ADP}{2(ATP + ADP + AMP)}$$

When compared to control values, treatment with amytal hardly affected the absolute purine nucleotide content although the values for the energy charge tended

Table 1
Metabolites and EC content in heart biopsies upon different treatments

	Treatment (in mg/kg body weight)		
	Control	Amytal 30 mg/kg b.wt.	Noradrenaline 0.02 mg/kg b.wt.
ATP	22 ±5	20.2 ±0.7	13.9 ±2.6
ADP	7 ±4	5.0 ±0.5	6.6 ±0.6
AMP	1.1 ±0.8	0.7 ±0.4	1.6 ±0.5
ATP/ADP	3.5 ±1.1	4.0 ±0.6	2.08±0.25
EC	0.86±0.03	0.877±0.025	0.78±0.03

Values (mean ± S.E.) are given in μmol/g

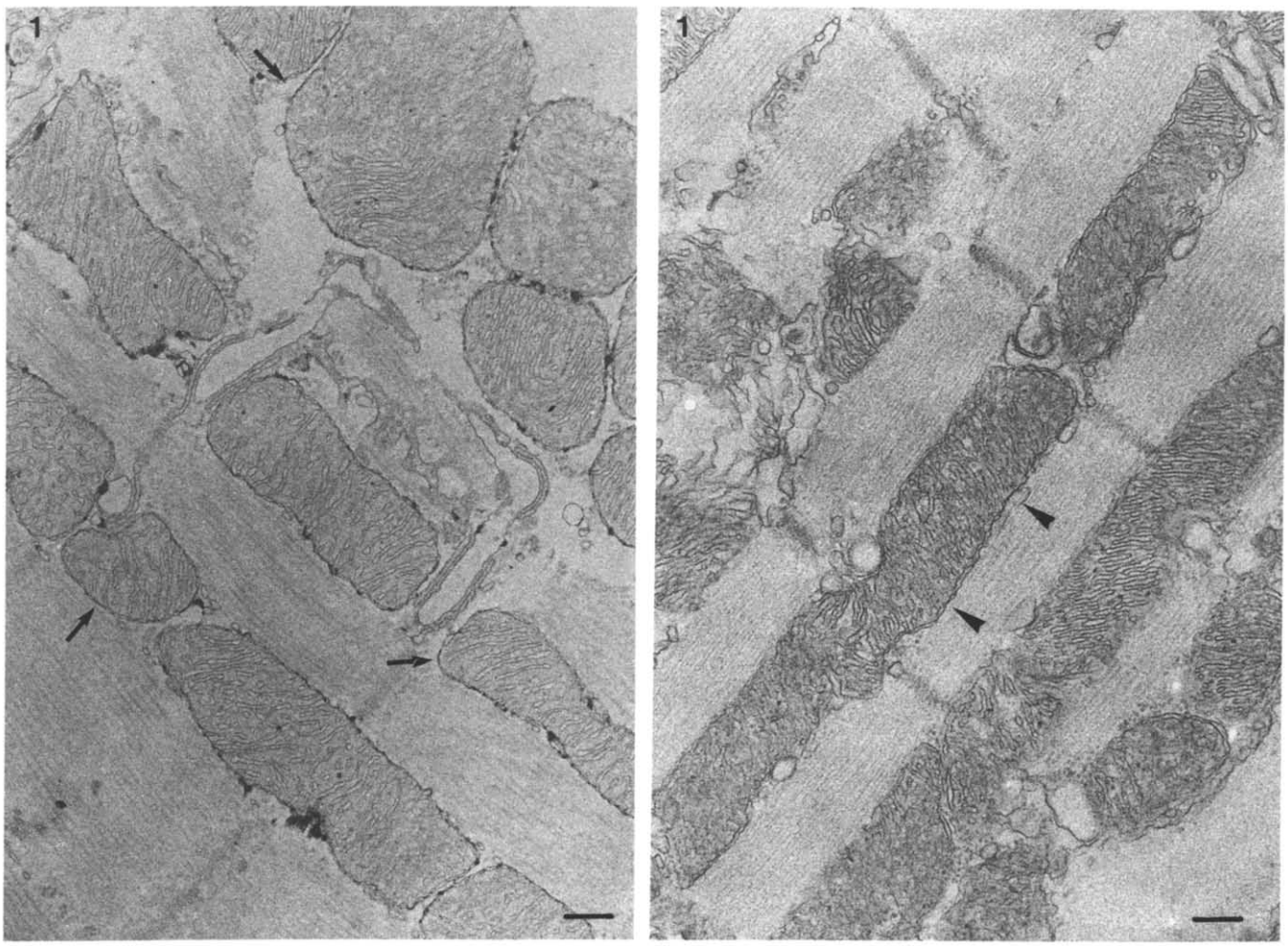

Fig. 1. Cytochemical CAT localisation in heart muscle of a stimulated animal. (A, left) Mitochondria in a section incubated in complete cytochemical medium, containing carnitine, acetyl CoA, $K_3Fe(CN)_6$ and uranylacetate. CAT activity, as shown by $UO_2\downarrow$ (= black precipitate) is localised in the contact sites (arrows). Bar = 250 nm. (B, right) Mitochondria in a section incubated in control medium without acetyl CoA and carnitine. The cytochemical deletion controls, obtained by omitting carnitine, acetyl CoA or carnitine and acetyl CoA, still contain contact sites (arrowheads) but no cytochemical precipitate could be shown. Bar = 250 nm.

to be somewhat higher. Noradrenaline induced a drop in ATP ($P \leq 0.05$ when compared to the combined values of controls and amytal) which is also reflected in the energy charge ($P \leq 0.05$)

Cytochemical data

Treatment with noradrenaline results in a close apposition of inner and outer mitochondrial membranes, so numerous contact sites are visible. An electrondense reaction product of CAT activity was cytochemically localised in these membrane contacts (Figs. 1A, 3A).

The electrondense reaction product is a result of the following reactions:

acetyl CoA + carnitine

$\longrightarrow$ acetylcarnitine + CoA SH

$2\ K_3Fe(CN)_6 + 2\ K^+ + \mathbf{2\ CoA\ SH}$

$\longrightarrow 2\ K_4Fe(CN)_6 + \mathbf{CoA\text{-}S\text{-}S\text{-}CoA} + 2\ H^+$

$2\ K_4Fe(CN)_6 + \mathbf{UO_2(C_2H_3O_3)_2}$

$\longrightarrow 2\ K_3Fe(CN)_6 + \mathbf{UO_2}\downarrow + 2\ K(C_2H_3O_3)$

black precipitate

In all cytochemical controls, i.e., sections of myocardia from the same animals, in media without the necessary substrates as acetyl CoA and carnitine, contact sites were still visible but without detectable precipitate (Figs. 1B, 3B).

Amytal, used as a partial inhibitor of the respiratory chain, resulted in a reduced number of contact sites, but CAT activity persisted in association with the remaining contacts (Fig. 2). Here also, cytochemical controls gave negative results.

Morphometrical data

The ratio of the surface density of CAT active contact sites to the surface density of the mitochondrial

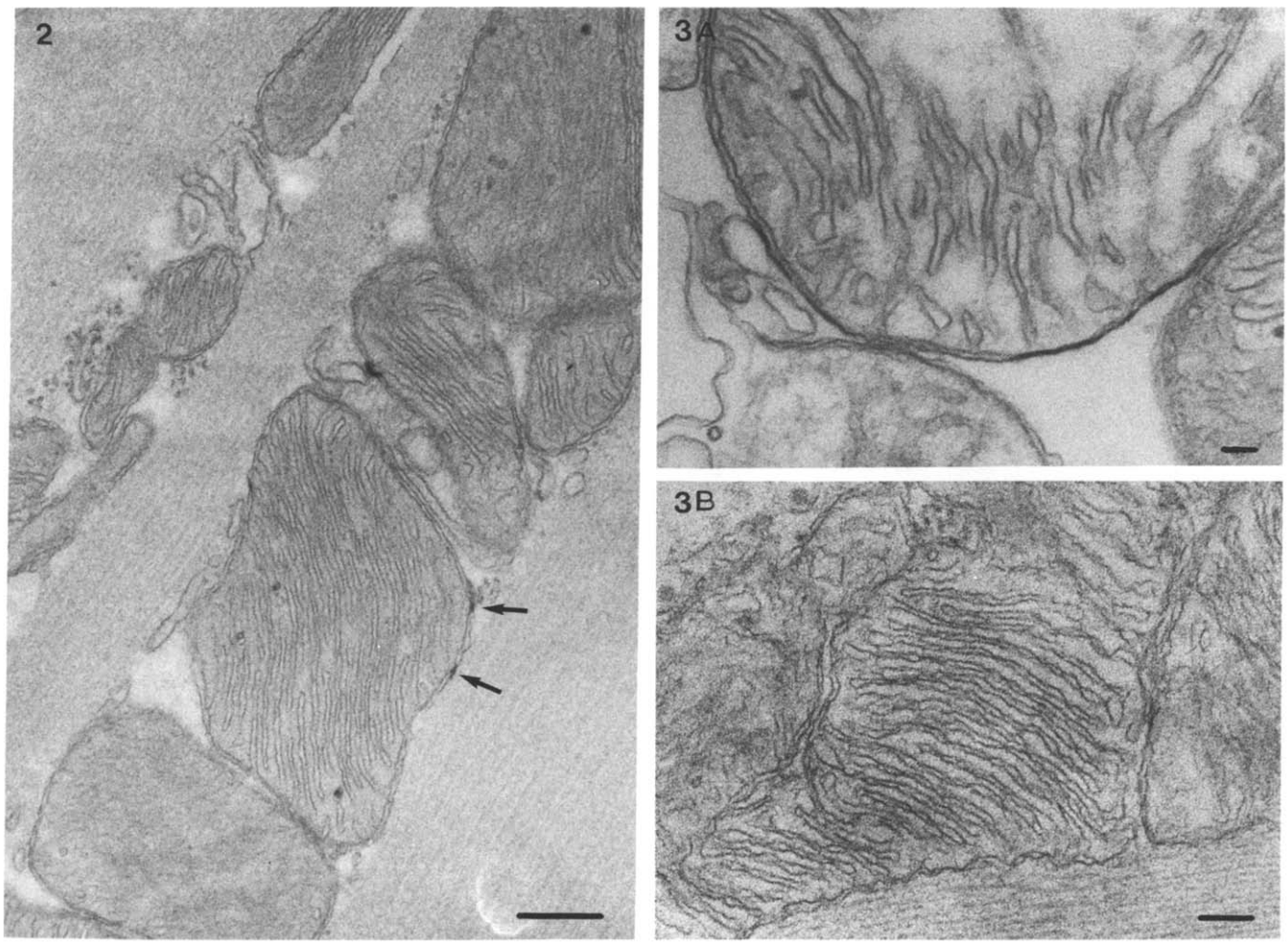

Fig. 2. Cytochemical CAT localisation in rat myocardium after treatment with amytal. In the remaining contact sites CAT activity is observed. Bar = 250 nm.

Fig. 3. A detail of the mitochondrial contact sites in sections of myocardium of a stimulated animal. (A) Incubation in complete cytochemical medium. Bar = 100 nm. (B) Incubation in the control medium which was obtained by omitting carnitine in the complete cytochemical medium. Bar = 100 nm.

Table 2
The ratio of the surface density of CAT active contact sites to the surface density of the mitochondrial membranes of rat hearts with different treatments

Treatment	$S_S \pm$ S.E.
Control	0.24 ± 0.06
Amytal	0.14 ± 0.02
Noradrenaline	0.29 ± 0.03

After anaesthesia with Nembutal, noradrenaline (0.02 mg/kg) and amytal (30 mg/kg) were injected via the vena femoralis. The control animals received only anaesthesia.

membranes is shown in Table 2. From these data it appears that amytal treatment markedly reduces this ratio when compared to control values ($P \leq 0.05$).

Noradrenaline increases the ratio, albeit that this effect is less pronounced than the drop of ATP. However, it should be kept in mind that in controls the sympathic tone may be increased by manipulation of the animals to a level sufficient to affect contact sites. Further stimulation by exogenous noradrenaline will lead to exhaustion of the nucleotides.

4. Discussion

In our rat model, on which we performed CAT cytochemistry, we considered different metabolic states characterised by different energy charges (EC). The value of the energy charge (EC) is indeed reflecting the energy status of the myocardium.

Treatment with for example noradrenaline causes such an increase in contractility and frequency of the heart, that the supply can no longer match the demand. Hence the energy charge drops. Treatment with amytal, which inhibits the first complex of the respiratory chain, provokes a decrease in heart rate and left ventricular pressure [14]. Under these different conditions, CAT activity was morphometrically quantified. Upon stimulation with noradrenaline the activity was increased, and treatment with amytal resulted in a decreased activity.

Up to now, explanations of the exact biochemical function of CAT have differed. Nevertheless, numerous biochemical data point to a significant role for this enzyme in the energy metabolism of myocardium, especially in mitochondrial oxidation of fatty acids [15–17] and pyruvate [18,19].

The general function of CAT is supposed to buffer against rapid changes in the acetyl CoA/CoA ratio in both the cytoplasmic and the mitochondrial compartments through the transport system of the carnitine shuttle [15,16]. This is controlled by the carnitine acetyl CoA transferase and the carnitine acyl carnitine translocase system of the inner mitochondrial membrane [20]. The acetyl CoA produced by β-oxidation can be used by the citrate cycle and, as an alternative route, mitochondrial acetyl can be disposed across the mitochondrial membrane to the cytosol where it is primarily stored as acetylcarnitine [16].

In the case of myocardial stimulation, CAT activity is increased but about the direction in which the acetyl is transported, from the mitochondrion to the cytosol or vice versa, is not yet clear.

According to Opie [21], during increased heart work, the mitochondria become more oxidised. The intramitochondrial level of $NADH_2$ and presumably $FADH_2$ fall and there is an increased turnover of the whole fatty acid oxidation spiral. Mitochondrial acetyl CoA falls as the citrate cycle accelerates and the cytosolic acetyl can enter the mitochondria after transformation to acetylcarnitine. Thereby free cytosolic CoA increases and the fatty acid activation is increased. [22]

However, during acute stress, as after noradrenaline treatment, the increasing pyruvate dehydrogenase activity and fatty acid supply from activated lypolysis may exceed the rate of acetyl CoA oxidation. As a consequence, a surplus of acetyl CoA is formed in the mitochondrial matrix [8,22]. A high mitochondrial acetyl CoA/CoA ratio is an effective inhibitor of pyruvate dehydrogenase [23] and of the β-oxidation [24]. So during this critical period, it is also possible that excess acetyl groups are converted into acetylcarnitine by CAT and transported across the mitochondrial membranes into the cytosol [8,18,22,25], making available more CoA SH in the matrix. The oxidation of pyruvate, α-ketoglutarate and fatty acids all depend on the availability of a common mitochondrial CoA SH pool.

In both ways of thinking, stimulation causes an increased CAT and translocase turnover. From the morphological point of view, within a few minutes after onset of the catecholaminergic stress situation, contacts between inner and outer mitochondrial membranes are promoted [9]. This will create an extending microcompartment for CAT activity. During reduced work load, as induced by partial inhibition of the respiratory chain, e.g. by amytal, the activity will be slowed down. This is illustrated by the results of our study where we did find an exclusive association of CAT activity with changing extents of contact sites between inner and outer mitochondrial membranes.

The mitochondrial localisation of CAT activity (in contact sites), and the correlation between the extent of CAT activity and the myocardial energetic state, are suggestive for an essential role of contact sites as an adaptive morphological mechanism during changing metabolic states, as already mentioned in previous investigations [9,10,26–28].

To summarise, we may conclude that these data demonstrate a strong correlation between contact sites and CAT activity. Our work reinforces the proposal by Murthy and Pandi [29], stating that sharing of a micro-

compartment between translocases and enzymes, e.g., creatine kinase and the ADP/ATP translocator and carnitine acetyltransferase and translocase, offers a distinct kinetic advantage during alterations in the cellular energy state of the heart.

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