Mitochondrial Contact Sites Detected by Creatine Phosphokinase Activity in the Hearts of Normal and Diabetic Rats: Is Mitochondrial Contact Sites Formation a Calcium-Dependent Process?

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Abstract. Mitochondrial contact sites (MiCS) are structures in the mitochondrial membrane containing the structure-bound mitochondrial isoenzyme of creatine phosphokinase that participates in the transfer of energy into the cytoplasm This explains the increased formation of MiCS found in hearts with high metabolic activity Earlier we demonstrated that enhanced MiCS formation may also be induced by perfusing the heart with increased, but still not cardiodepressive concentrations of Ca^{2+} (2 2 mmol l^{-1}) in the perfusate Nevertheless, neither the molecular mechanism by which Ca^{2+} ions may induce an increase in MiCS formation, nor the dependence of Ca^{2+}-induced MiCS formation on the intracellular Ca^{2+} level have yet been elucidated In the present study we investigated the effect of Langendorff-perfusion with 2 2 mmol l^{-1} Ca^{2+} on formation of MiCS in normal as well as in diabetic hearts The latter, namely, are characterized by altered metabolism as well as Ca^{2+}-handling, resulting in elevated $[Ca^{2+}]_i$ We have found that the amounts of MiCS in diabetic hearts outnumbered those in normal hearts Our results showed that in comparison to perfusion with 1 6 mmol l^{-1} Ca^{2+} a perfusion with 2 2 mmol l^{-1} Ca^{2+} is capable of significantly increasing ($p < 0$ 01) the formation of MiCS in control hearts In both groups of diabetic hearts the numbers of MiCS were significantly increased in comparison to healthy controls ($p < 0$ 01) Moreover, no significant differences in amounts of MiCS were found between healthy hearts perfused with 2 2 mmol l^{-1} Ca^{2+} and diabetic hearts of both groups ($p > 0$ 05) In diabetic hearts, MiCS formation in response to $[Ca^{2+}]_e$ was little manifested Our results also confirmed that elevated $[Ca^{2+}]_i$ in all cases represented a signal for increased formation of MiCS in the heart

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Introduction

Creatine phosphokinase (CPK) is an essential enzyme of the intracellular energy transport chain having particular importance in tissues and cells with high energy demands, such as heart, skeletal muscle, brain etc (Wallimann et al 1986) Muscle CPK exhibits specific isoforms localized in the cytoplasm, on myofibrils and in the mitochondria Among them special attention requires the mitochondrial isoenzyme that occurs in the mitochondrial contact sites (MiCS) MiCS are entities consisting of porine in the outer membrane, the octameric mitochondrial creatine phosphokinase (MiCPK) in the inter-membrane space, and the ATP/ADP translocase of the inner mitochondrial membrane MiCS are believed to participate in transfer of energy and proteins through the mitochondrial membranes (Brdiczka 1991 Wyss et al 1992) MiCS were first demonstrated ultrastructurally by Hackenbrock (1968) Cytochemical detection of MiCS, based on the presence of MiCPK octameres, was performed by Bieimans et al 1989 These autors revealed that MiCS are more abundant when the metabolic activity of the heart increases In our previous study we have reported that enhanced MiCS formation may also be induced by perfusion of the isolated rat heart with increased (not cardiodepressive) concentrations of Ca^{2+} in the perfusate (Bakker et al 1994) This finding indicated that an increase in MiCS formation may result from changes in intracellular Ca^{2+}-transients, depending on influx of extracellular Ca^{2+} ions However, the molecular mechanisms that may couple intracellular Ca^{2+}-transients with the formation of MiCS still remain to be elucidated In any case, it may be anticipated that different basal levels of intracellular free calcium might facilitate or inhibit the transmission of the external Ca^{2+}-signal to the site of formation of MiCS In the present study we focused on the investigation of the latter assumption For this reason we studied the effect of Langendorff-perfusion with an elevated Ca^{2+} concentration ($[Ca^{2+}]_e$, 2 2 mmol l^{-1}) on the formation of MiCS in normal as well as in diabetic hearts Diabetic hearts were chosen intentionally because they are characterized by elevated free Ca^{2+} levels in the cytoplasm ($[Ca^{2+}]_i$) as a result of altered metabolism as well as membrane functions (Ravingerová et al 1996, Ziegelhoffer et al 1996) Perturbations in sarcolemmal Ca^{2+}-binding, Na,K-ATPase activity and calcium pump activity as well as disturbed sarcoplasmic reticular Ca^{2+}-binding and Ca^{2+}/Mg^{2+}-ATPase activity, etc are all involved in malfunction of membrane systems All these are believed to participate in the impairment of Ca^{2+}-handling in the myocytes, and result in diminished quality of relaxation (Rodrigues and McNeill 1992)

Materials and Methods

Experimental protocol

Experiments were performed in adult male Wistar rats fed standard pellet diet and having free access to water Diabetes was induced by venous injection of streptozotocin (STT) in a single dosis of 45 mg/kg b wt Beginning with the first day after STT treatment the animals also obtained insulin (Interdep), 6 U i m daily After control of the metabolic state (glucose, triglycerides and cholesterol in the blood, Table 1) on day 8 following STT administration, the rats were sacrificed Age matched controls were running with all experimental groups

Perfusion of isolated rat hearts

Hearts were quickly excised and perfused for 15 min at a pressure of 75 mm Hg and 37 °C, using the Langendorff technique without recirculation of perfusate Krebs-Henseleit buffer containing (in mmol l^{-1}) 118 5 NaCl 25 0 $NaHCO_3$, 1 2 KH_2PO_4, 1 18 KCl, 1 2 $MgSO_4$, 11 5 glucose, and either 1 6 (normal Ca^{2+} content) or 2 2 (increased Ca^{2+} content) $CaCl_2$ was used as perfusion medium, it was gassed with a mixture of 95% oxygen and 5% carbon dioxide A stabilization perfusion lasting 15 min (SP) with medium containing 1 6 mmol l^{-1} Ca^{2+} preceded all experiments The technique of perfusion was essentially similar to that described in Ravingerová et al (1996) After termination of the perfusion the hearts were further processed for cytochemical determination of MiCS

Experimental groups

Hearts were divided in 4 groups Group 1 – hearts from healthy control rats, perfused after SP with 1 6 mmol l^{-1} Ca^{2+}, Group 2 – hearts from healthy rats, perfused after SP with 2 2 mmol l^{-1} Ca^{2+} (high Ca^{2+} – rats), Group 3 – hearts from diabetic rats, perfused after SP with 1 6 mmol l^{-1} Ca^{2+} (diabetic control rats) and Group 4 – hearts from diabetic rats, perfused after SP with 2 2 mmol l^{-1} Ca^{2+} (high Ca^{2+}-diabetic rats)

Cytochemical detection of creatine phosphokinase

Hearts of all groups were fixed for 10 min in 2% formaldehyde in 50 mmol l^{-1} cacodylate buffer (pH 7 2), containing 10% glucose, 6% dextrane as well as cystein 10 mmol l^{-1}, and subsequently cryoprotected with 50 mmol l^{-1} cacodylate buffer (pH 7 2) containing 10% glucose for 10 min Subsequently, hearts were cut in small pieces and further cryoprotected, first with 50 mmol l^{-1} cacodylate buffer (pH 7 2) containing 10% glucose for 30 min and then with 7% dimethylsulfoxide for additional 30 min After cryoprotection tissue pieces were frozen at – 70 °C and cut in the cryostate (Karl-Zeiss, Jena) to 40 μm thick slices

Cytochemical detection of creatine phosphokinase was performed using the method of Biermans et al (1989) Slices were preincubated for 20 min with 50 mmol l^{-1} Tris buffer (pH 7 2) containing (in mmol l^{-1}) 1 ADP, 15 AMP, 330 glucose, 10 magnesium acetate, 30 U ml^{-1} hexokinase, 0,150 U ml^{-1} glucose-6-phosphate dehydrogenase, 0 6 thiocarbamyl nitroblue tetrazolium salt, and 1 3 phenazine methosulphate Preincubation was followed by incubation for 60 min with the same solution containing in addition 1 mmol l^{-1} NADP and 6 4 mmol l^{-1} creatine phosphate Following the incubation, slices were 3 times rinsed in 50 mmol l^{-1} cacodylate buffer (pH 7 2), postfixed for 45 min in 1% osmium tetroxide at 40 °C, and further processed using standard electron microscopic techniques Controle incubations were run in parallel with all experiments for detection of creatine phosphokinase i) in the absence of creatine phosphate in the incubation medium ii) in the presence of creatine phosphokinase inhibitor 1 flouro-2,4 dinitrobenzene (2 mmol l^{-1})

Thin sections of embedded tissue slices were examined in a Tesla 500 electron microscope

For the numerical evaluation of the amount of MiCS we measured the area of mitochondrial sections and evaluated the amounts of MiCS per section area unit Student s *t*-test was used for statistical evaluation

All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No 85 23 revised 1985)

Results

Diabetic rats were characterized by decreased heart-to-body weight ratio The levels of glucose, triglycerides and cholesterol in the blood are given in Table 1

Table 1. Blood levels of glucose, triglycerides and cholesterol in rats with acute diabetes

	Healthy rats	Rats with acute diabetes
Glucose	5 66 mmol l^{-1}	17 10 mmol l^{-1}
Triglycerides	1 20 g l^{-1}	4 58 g l^{-1}
Cholesterol	1 80 g l^{-1}	2 40 g l^{-1}

Electrone microscopic investigation of tissue specimens from hearts of control animals perfused with 1 6 mmol l^{-1} of Ca^{2+} exhibited relatively scarce cytochemical reaction for MiCPK (Fig 1) MiCS became all stained, although they were not

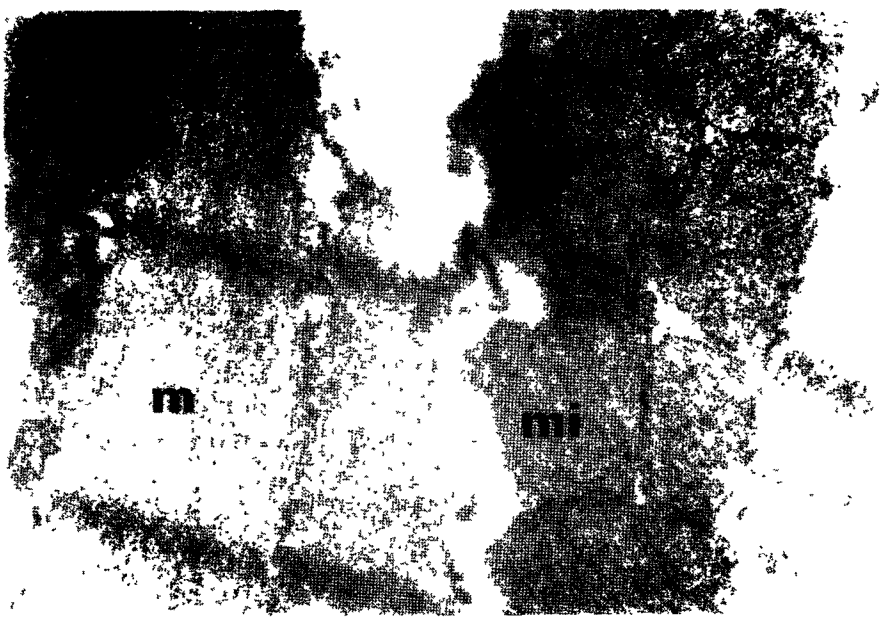

Figure 1. Creatine phosphokinase activity in contact sites on mitochondrial membranes from healthy control rat heart (mi – mitochondria, m – myofibrills, arrows – contact sites 35,000 ×)

very abundant In negative controls, where the reaction for CPK was performed either in the absence of substrate or in the presence of CPK inhibitor, several loci could be identified, where the inner and outer Mi membranes were in close opposition, but the cytochemical reaction product was missing Same type of negative controls was also revealed in all other groups investigated.

An increase in contractility of about 20% in the group of healthy hearts perfused with elevated $[Ca^{2+}]_e$ indicates stimulation of the hearts via increased Ca^{2+} transients Also in this group (Group 2) all MiCS were stained (Fig. 2), and their amounts were increased considerably ($p < 0.01$). This finding may be considered as a sign of Ca^{2+}-induced enhancement in MiCS formation. In comparison with control hearts (Group 1), diabetic hearts, with high $[Ca^{2+}]_i$, correspondingly revealed increased numbers of MiCS. This finding was manifested expressively ($p < 0.01$) without any triggering by $[Ca^{2+}]_e$, i. e. already in hearts perfused with the medium containing 1.6 mmol.l^{-1} Ca^{2+} only (Group 3; Fig. 3). It is noteworthy that the amounts of MiCS in Group 3 (diabetic controls) were not significantly different ($p > 0\ 05$) from increased amounts of MiCS in Group 2 (high Ca^{2+} – rats) In diabetic hearts perfusion with elevated $[Ca^{2+}]_e$ did not induce any changes in con-

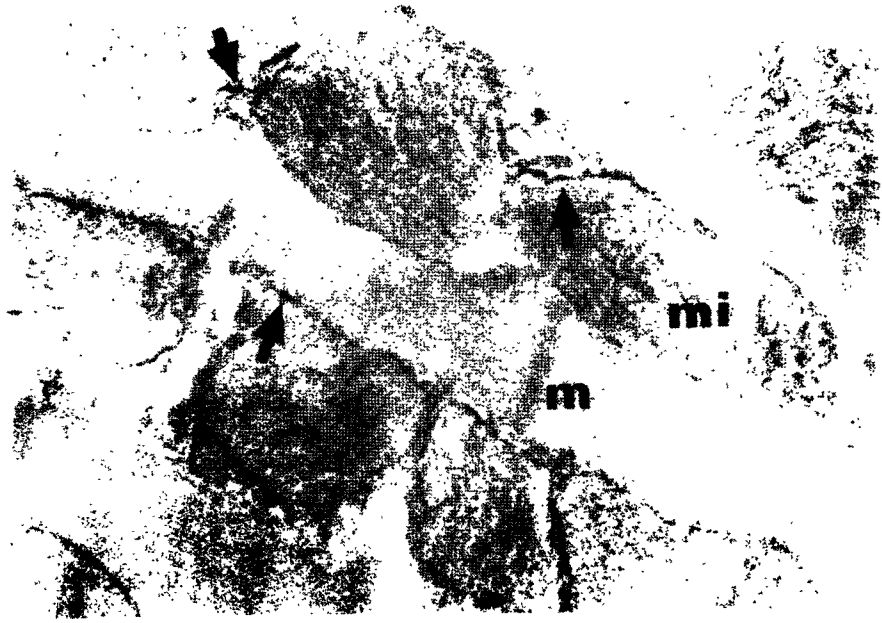

Figure 2. Contact sites on mitochondrial membranes in healthy rat heart perfused with 2.2 mmol.l^{-1} calcium, detected by creatine phosphokinase activity (mi – mitochondria; m – myofibrills, arrows - contact sites; 45,000 ×)

Figure 3. Contact sites on mitochondrial membranes in control diabetic rat heart, detected by creatine phosphokinase activity (mi – mitochondria; m – myofibrills; arrows – contact sites, 46,000 ×).

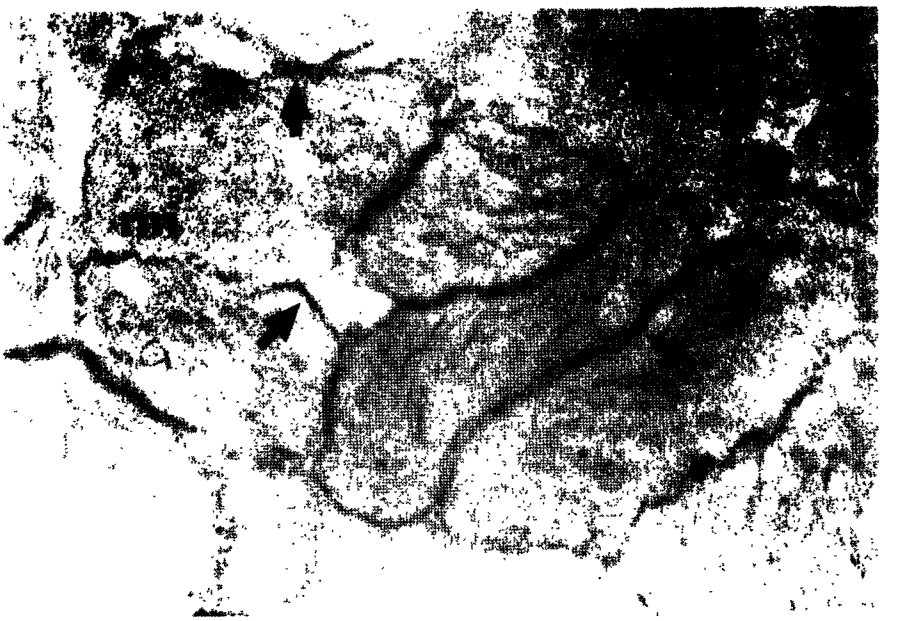

Figure 4. Creatine phosphokinase activity in contact sites on mitochondrial membranes from diabetic rat heart, perfused with 2.2 mmol.l^{-1} calcium (mi – mitochondria, m – myofibrills; arrows – contact sites; 86,000 ×).

Table 2. Amounts of MiCS per mitochondrial section unit area

	Healthy controls	Healthy high-calcium rats	Diabetic controls	Diabetic high-calcium rats
MiCS/cm^2 ± S.D.	3.5 ± 0.57	12.9 ± 2.9	12.5 ± 4.2	14.5 ± 3.6

tractility, pressure or heart rate. Even more the elevated $[Ca^{2+}]_e$ was not able to produce any signifficant increase in the amount of contact sites ($p > 0.05$) in the cardiac mitochondria of the diabetic animals (Group 4; Fig. 4). Similarly to healthy hearts, all contact sites present in the hearts of diabetic animals became stained with the precipitated product of creatine phosphokinase reaction.

The differences in areas of mitochondrial sections were insignificant ($p > 0.05$) for all experimental groups. But there were significant differences in the number of MiCS per unit (cm^2) of mitochondrial section area. We observed a significant increase ($p < 0.01$) in the value of MiCS/cm^2 for groups of healthy high-calcium

rats, diabetic control rats and diabetic high-calcium rats in comparison to healthy control rats The values of MiCS/cm^2 for Groups 1–4 are shown in Table 2

Discussion

The transport of energy from cardiac mitochondria to the cytoplasm that occurs via MiCPK is related to energy demands of the heart (Ziegelhoffer et al 1982, Knoll and Brdiczka 1983) and it seems to represent a sequence of events modulated by a signal which might be propagated by Ca^{2+} ions This hypothesis gets support also from the knowledge that Ca^{2+} ions are crucial for the regulation of both, the production of ATP in mitochondria and its intracellular transport, as well as for excitation-contraction coupling in the myocardium (Dhalla et al 1996) Moreover, it is believed that calcium is an important signal-trigger for many intramitochondrial enzymes participating in the energy production (Račay and Lehotský 1996)

The hypothesis about the modulation of MiCS formation in cardiac mitochondria by Ca^{2+} has already been partly proved by Bakker et al (1994) and our selves (Ziegelhoffer et al 1995) Our experimental results presented in this study confirmed again the formation of significant amounts of MiCS that occur upon increased Ca^{2+} supply to the heart Although the mechanism of increased MiCS formation is triggered by elevation of $[Ca^{2+}]_e$, in subsequent phases, changes in $[Ca^{2+}]_i$ will play an essential role, i e $[Ca^{2+}]_e$ induced oscillation of free $[Ca^{2+}]_i$ in the cytoplasm of cardiac cells (Poizat et al 1996) In this respect, the intensity of the intracellular Ca^{2+} signal that appears in response to a similar extracellular Ca^{2+}-impulse will strongly depend on basal $[Ca^{2+}]_i$ in the cardiac myocytes We have hypothesized that the basal $[Ca^{2+}]_i$ itself might act in regulating the actual amounts of MiCS present in the heart cells This assumption was tested in our experiments with diabetic hearts which are well known to exhibit constantly elevated levels of $[Ca^{2+}]_i$ The results obtained brought evidence about increased numbers of MiCS formed spontaneously, without any induction by $[Ca^{2+}]_e$, already in the diabetic controls (Table 2) The numbers of MiCS detected in the hearts of diabetic animals were comparable with those found in healthy hearts perfused with elevated calcium Our results indicated also, that the formation of MiCS achieved upon perfusion with elevated $[Ca^{2+}]_e$ (2 2 mmol l^{-1} Ca^{2+}), was close to maximal because further elevation of $[Ca^{2+}]_e$ in perfusate already had cardiodepressive effect and provided no additional MiCS (Bakker et al 1994, Ziegelhoffer et al 1995) The same seems to concern also spontaneous MiCS formation in hearts of diabetic animals, since perfusion of these hearts with elevated $[Ca^{2+}]_e$ was no more effective in inducing further formation of MiCS in considerable numbers From our results it may be concluded that 1) Ca^{2+} ions act as a signal for on-demand augmentation of energy transport through the membranes of cardiac mitochondria This was proven by the finding of significantly elevated amounts of MiCS detected in healthy hearts

with $[Ca^{2+}]_i$ increased upon their perfusion with elevated $[Ca^{2+}]_e$ ii) The basal $[Ca^{2+}]_i$ in cardiac myocytes itself has a regulatory role in maintaining the actual amounts of MiCS on the required level This was proven in hearts of diabetic animals which showed elevated $[Ca^{2+}]_i$ and consequently exhibited increased numbers of MiCS

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