

Calcium signaling-mediated endogenous protection of cell energetics in the acutely diabetic myocardium¹

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Abstract: In acute diabetic myocardium, calcium signals propagated by intracellular calcium transients participate in the protection of cell energetics via upregulating the formation of mitochondrial energy transition pores (ETP). Mechanisms coupling ETP formation with an increase in membrane fluidity and a decrease in transmembrane potential of the mitochondria are discussed. Our results indicate that the amplification of calcium transients in the diabetic heart is associated with an increase in their amplitude. Moreover, the signals transferred by calcium transients also regulated ETP formation in nondiabetic myocardium. Evidence for the indispensable role of calcium in the regulation of transition pore formation is provided whereby an exchange of cadmium for calcium ions led to a rapid and dramatic decrease in the amount of ETP. Another possible regulatory factor of the mitochondrial function may be radical-induced damage to the diabetic heart. Nevertheless, our data indicate that radical-induced changes in mitochondria predominantly concern the respiratory chain and have no appreciable effect on the fluidity of the mitochondrial membranes. The residual mitochondrial production of ATP owing to its augmented transfer to the cytosol proved to be adequate to preserve sufficient levels of adenine nucleotides in the acute diabetic myocardium.

Key words: myocardium, streptozotocin diabetes, mitochondria, energy transition pores, calcium transients, calcium signaling, membrane fluidity, transmembrane potential, energy metabolism.

Résumé : Les signaux calciques propagés par les transitoires calciques intracellulaires participent à la protection de l'énergie cellulaire dans le myocarde diabétique aigu, par la régulation positive de la formation des pores de transition d'énergie (PTÉ) dans les mitochondries. Nous discutons des mécanismes couplant la formation des PTÉ avec une augmentation de la fluidité membranaire et une diminution du potentiel transmembranaire des mitochondries. Nos résultats indiquent que l'amplification des transitoires calciques dans le cœur diabétique est associée à une augmentation de leur amplitude. De plus, les signaux transportés par les transitoires calciques régulent la formation des PTÉ dans le myocarde non diabétique. Le rôle crucial du calcium dans la régulation de la formation des pores de transition est démontré dans l'expérience, où une substitution de cadmium aux ions calcium a entraîné une diminution rapide et spectaculaire de la quantité des PTÉ. Un autre facteur possible de la régulation de la fonction mitochondriale pourrait être l'altération du cœur diabétique par les radicaux. Nos données indiquent que les modifications induites par les radicaux dans les mitochondries touchent principalement la chaîne respiratoire et qu'elles n'ont pas d'effet significatif sur la fluidité des membranes mitochondriales. La production mitochondriale résiduelle d'ATP, en raison de son transfert accru au cytosol, a été suffisante pour préserver les taux d'adénonucléotides dans le myocarde diabétique aigu.

Mots-clés : myocarde, diabète induit par streptozotocine, mitochondries, pores de transition d'énergie, transitoires calciques, signalisation calcique, fluidité membranaire, potentiel transmembranaire, métabolisme énergétique.

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Introduction

The fact that calcium signaling plays an important role in the regulation of cardiac energy metabolism in health and disease is well studied and widely understood. Nevertheless, there are still aspects of calcium signaling that require further elucidation. This is particularly true of the role of calcium-modulated mechanisms associated with adaptation of the myocardium to diverse pathologic impulses. These impulses may have limited duration, such as ischemia–reperfusion, temporary hypoxia, or different types of preconditioning (Ziegelhöffer et al. 1995; Ylitalo et al. 2000; Ravingerová 2005). However, adaptation is also experienced in response to pathological impulses persisting for long periods of time, like chronic hypoxia (Strnisková et al. 2006) or diabetes (Ziegelhöffer 2005). In diabetes, the benefits originating from the processes of endogenous protection, which lead to adaptation, appear to be much more expressed in the early and acute phase of the disease (Ziegelhöffer et al. 2002; Ferko et al. 2006, 2008) than later, in the advanced phase of diabetes, in which adaptation benefits gradually become overlapped with permanently accumulating damage to diverse subcellular processes and structures (Pierce and Dhalla 1984; Severson et al. 2003). Common features of the aforementioned pathologic mechanisms leading to adaptation are increased calcium signaling, augmented intracellular calcium transients, and perturbations in the intracellular calcium handling. These features may yield calcium overload of the cardiac cells that can be either strongly or weakly catecholamine-dependent or -independent. (Götzsche 1991; Kawamura and Suzuki 1991; Di Lisa et al. 1995; Ziegelhöffer et al. 1997).

In pathological situations, calcium signals mediate multiple effects in cardiac energy metabolism. They participate in the decline in oxidative energy production in the mitochondria (Kuo et al. 1983; Pierce and Dhalla 1984; Ferko et al. 2006) and, at the same time, they stimulate energy consumption by most cellular ATPases (Seymour and Brosman 1991; Mujkošová et al. 2008). Strong calcium signals may therefore induce a strained situation in energy metabolism of the myocardium. On the other hand, calcium signals also increase cardiac contractility and cardiac output, thus also indirectly increasing coronary blood supply, which would be a positive effect. In addition, augmented calcium transients were shown to induce increased formation of energy transition pores, also termed energy channels or membrane contact sites, across the mitochondrial membranes. These ephemeral energy transition pores enable high-capacity transfer of energy from the mitochondria to the cytoplasm (Bakker et al. 1994) and may compensate for reduced production of ATP by its improved delivery and availability in myocardial cells.

Energy transition pore formation, even if fast, is a relatively complicated process with a still-undisclosed mechanism of initiation by calcium. It involves a series of protein–protein and (or) protein–lipid interactions, not as yet satisfactorily characterized, which are coupled with considerable changes in the physical and structural properties of the mitochondrial membranes (Ziegelhöffer–Mihalovicová et al. 1997). These questions also concern the process of decay of the energy transition pores.

In this study we present a new hypothesis based on data already published as well as on new original experiments concerning the regulation of mitochondrial membrane fluidity by energy transition pore formation. We suggest that changes in the physical state of mitochondrial membranes may participate as an endogenous protective mechanism in diverse pathological situations and may be particularly significant in acute diabetic rat heart (Ziegelhöffer et al. 2002).

Materials and methods

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health) as well as the rules issued by the State Veterinary and Alimentary Administration of the Slovak Republic, based on paragraph 37 (6), legislation No. 488/2002 of the Slovak Parliament.

Animals

All experiments were performed on adult male Wistar rats (220 ± 10 g). Animals were randomly allocated to either control or diabetic groups with $n = 120$ animals in each. Both groups were divided into 4 subgroups: one for isolation of cardiomyocytes with the goal to investigate the calcium transients, a 2nd for isolation of heart mitochondria, a 3rd for estimation of energy transition pores, and a 4th for estimation of high-energy phosphate content in cardiac tissue. Animals were kept under a standard 12 h light : 12 h dark regimen at $22 \pm 2^\circ\text{C}$. They were fed a standard pellet diet and had free access to water.

Induction and control of experimental diabetes

Diabetes was induced in rats by intraperitoneal administration of streptozotocin in a single dose of $65 \text{ mg}\cdot\text{kg}^{-1}$ body weight. Manifestation of the disease was followed daily by estimation of glucosuria using Gluko Phan strips (Pliva-Lachema, Brno, Czech Republic) and confirmed by estimation of glucose (BIO-LA-TEST, glucose oxidase (GOD250), Pliva-Lachema) and glycohemoglobin (Burrin et al. 1980) in blood as well as insulin in serum (commercial Ria kit, Linco Research). The biochemical estimations were made at the beginning and end of the experiment. The experiment was terminated on the 8th day after streptozotocin application.

Isolation of cardiomyocytes

Animals were anesthetized with thiopental (Valeant, Czech Pharma, $45 \text{ mg}\cdot\text{kg}^{-1}$) in the presence of heparin (Zentiva, Léčiva, $100 \text{ mg}\cdot\text{kg}^{-1}$). After bilateral thoracotomy the hearts were quickly excised, cannulated, and perfused retrogradely through the aorta at a pressure of 700 mm H₂O with approximately 400 mL of a basic solution containing (in $\text{mmol}\cdot\text{L}^{-1}$) 130 NaCl, 5.4 KCl, 1.4 MgCl₂·6H₂O, 0.4 NaH₂PO₄, 10 taurine, 10 glucose, and 10.0 HEPES, pH 7.4, supplemented with $0.75 \text{ mmol}\cdot\text{L}^{-1}$ CaCl₂. Then a similar perfusion followed with the same basic solution in which CaCl₂ was replaced by $0.1 \text{ mmol}\cdot\text{L}^{-1}$ EGTA. The procedure was finished with a 3rd perfusion with the basic solution supplemented with $0.9 \text{ mg}\cdot\text{mL}^{-1}$ collagenase (type II, Gibco), $0.01 \text{ mg}\cdot\text{mL}^{-1}$ protease (type XIV, Sigma-Aldrich), and $50 \mu\text{mol}\cdot\text{L}^{-1}$ CaCl₂ at 36°C . This perfusate was col-

lected and the batches of myocytes were centrifuged at 50g for 1 min. The pellet containing isolated cardiomyocytes was then resuspended and stored in an enzyme-free basic solution also containing 0.75 mmol·L⁻¹ CaCl₂. Only rod-shaped cells with clearly defined sarcomere striations and with no blebs were taken for measurements of calcium transients. All experiments were performed within 10 h of isolation.

Potassium chloride, CaCl₂, and glucose were purchased from Merck; NaCl, MgCl₂, NaH₂PO₄, thiopental, and heparin were from Lachema (Czech Republic). For more details to this part, see Chorvátová et al. (2004).

Isolation of mitochondria

After application of heparin and anesthesia as described above, hearts were quickly excised and cooled down and washed free of blood with ice-cold saline solution. After removal of vessels and fat, hearts were weighed, moistened with a small volume of ice-cold isolation solution (containing 180 mmol·L⁻¹ KCl, 4 mmol·L⁻¹ EDTA, and 1% bovine serum albumin, pH 7.4) and subsequently cut into small pieces with scissors. The minced tissue was then transferred to a teflon glass homogenizer together with 20 mL of the isolation solution also containing protease (Sigma P-6141) 2.5 mg·g⁻¹ (heart wet weight) and homogenized gently for 2–3 min. After centrifugation at 1000g for 10 min, the protease-containing supernatant was discarded together with a part of mitochondria being in direct contact with the protease. The pellet was resuspended in the same volume of the protease-free isolation solution. This supernatant, now mostly containing mitochondria that were not in direct contact with protease, was spun down at 5000g for 15 min. The pellet containing mitochondria was again resuspended in an albumin-free isolation solution containing only 180 mmol·L⁻¹ KCl and 4 mmol·L⁻¹ EDTA, spun down at 5000g for 15 min, and subsequently used for the estimation of functional parameters and membrane fluidity. The whole isolation procedure was performed at 4 °C.

Because of considerable seasonal differences in the functional properties and some enzyme activities of the cardiac mitochondria (Mujkořová et al. 2008), all experiments with isolated mitochondria were performed in seasonal blocks not exceeding 4–6 weeks. Protein concentration was determined according to Lowry et al. (1951).

Estimation of functional parameters of the mitochondria

Functional parameters of isolated mitochondria (state 3 and 4 oxygen consumption, respiratory control index, oxidative phosphorylation rate, and ADP:O ratio) were estimated by means of the Clark oxygen electrode polarized to 8 V (Oxygraph Gilson) at 30 °C using either succinate or glutamate + malate as substrates. The incubation medium contained (in mmol·L⁻¹) 3 KH₂PO₄, 120 KCl, 0.5 EDTA, 12.5 Hepes, 0.5 substrate (either succinate or glutamate + malate), and 2% dextran. ADP at a concentration of 500 nmol·L⁻¹ was used as a phosphate acceptor. The reaction was started by addition of the mitochondria (1 mg·mL⁻¹). All the chemicals applied, with the exception of KH₂PO₄ and KCl (Lachema, Czech Republic), were purchased from Sigma-Aldrich. For more details see Ferko et al. (2006, 2008).

Estimation of membrane fluidity of the mitochondria

Fluidity of the lipid layer of the mitochondrial membrane was assessed by measuring steady-state fluorescence anisotropy of a lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich). The isolated mitochondria were resuspended at a final protein concentration of 0.5 mg·mL⁻¹ in an isotonic buffer (containing 180 mmol·L⁻¹ NaCl and 4 mmol·L⁻¹ EDTA, adjusted to pH 7.4) and labelled with an equal volume of DPH dissolved in the isotonic buffer at a concentration of 0.5 μmol·L⁻¹. The final concentration of the probe in the sample was 0.25 μmol·L⁻¹. Samples were incubated at 22 ± 1 °C for 20 min to allow complete incorporation of the probe into the membranes. Steady-state fluorescence anisotropies (Shinitzky 1984) were measured at 22 ± 1 °C with a Perkin-Elmer LS45 luminescence spectrometer. Fluorescence excitation was set at 360 nm (10 nm slit width) and emission was detected at 425 nm. The degree of fluorescence anisotropy was estimated as described previously by Waczulíková et al. (2007).

Estimation of conjugated dienes

Content of conjugated dienes in the lipids from mitochondrial membranes was assessed by the method of Kogure et al. (1982) adapted to the estimation of conjugated dienes in the membranes of heart mitochondria. The adaptation concerned the adjustment of optimal conditions for extraction of the membrane lipids. Briefly, freshly isolated heart mitochondria were suspended at a concentration of 1 μg·mL⁻¹ in a solution containing 180 mmol·L⁻¹ KCl and 4 mmol·L⁻¹ Na₂EDTA adjusted to pH 7.4 by Tris-HCl. Lipids were extracted from 500 μL of the membrane suspension with 1000 μL of chloroform:methanol (1:2 v/v) under vortexing for 30 s. Subsequently, 500 μL of chloroform was added and vortexed again for 30 s. Extraction was terminated by addition of 500 μL of 15 mmol·L⁻¹ Na₂EDTA containing 4% NaCl and spinning down for 10 min at 1900g. Then 600 μL of the lipid-containing lower layer of chloroform:methanol was transferred to a separate test tube and carefully evaporated at room temperature under a continuous stream of nitrogen to prevent oxidation. Beginning from this point, all the procedure was performed in a nitrogen atmosphere. The dry lipids were then dissolved in 3 mL of cyclohexane, vortexed for 30 s, and used directly for spectrophotometric determination of the conjugated dienes at λ = 233 nm, ε = 29 000 L·mol⁻¹·cm⁻¹.

Estimation of calcium transients by fluorescence microscopy

Isolated ventricular myocytes were stained with the calcium indicator fluo-3 (fluo-3 acetoxymethyl ester, 1 μmol·L⁻¹; Molecular Probes, Eugene, USA) at room temperature for 20 min. Cells were allowed to settle on a cover slip, enabling perfusion at a rate of 1 mmol·L⁻¹·min⁻¹, and they were stimulated via platinum electrodes (4 ms, 5 V·cm⁻¹). Composition of the perfusion medium was similar to that used for the isolation of the cardiomyocytes. All recordings were carried out at room temperature. Fluorescence was collected with filter set 09 (excitation 470 nm, emission 515 nm, respectively) using the fluorescence microscope Axiovert 200 (Zeiss) equipped with an oil immersion objective (40×, NA 1.3) and a Hg lamp for excitation. The fluores-

cence image was amplified by a multichannel plate-based intensifier unit (Deltatekh, Russia) and detected by a standard black and white video camera at 25 frames per second. Calcium transient amplitude was defined as a ratio of peak (systolic) and resting (diastolic) fluo-3 fluorescence intensity collected from the whole area of single cardiomyocytes originating from 11 independent experiments with a total of 51 cells from nondiabetic hearts and 55 cells from diabetic hearts.

Estimation of intracellular calcium concentrations

Fluo-3 was dissolved in DMSO and incubated at room temperature for 20 min to allow the dye to de-esterify. For the measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$), isolated cardiomyocytes were loaded with $1 \mu\text{mol}\cdot\text{L}^{-1}$ fluo-3. The final concentration of dimethyl sulfoxide was $<0.1\%$. Fluorescence was collected as described above.

We performed fluo-3 calibration of $[Ca^{2+}]_i$ and a heavy metal, using the method according to Kao et al. (1989). In brief, a maximal Ca^{2+} saturation of the fluorescence dye in the cardiac cells was achieved using Ca^{2+} ionophore ionomycin ($2.5 \mu\text{mol}\cdot\text{L}^{-1}$; Sigma) in a solution (indicated as "A") containing (in $\text{mmol}\cdot\text{L}^{-1}$) 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 Hepes (pH 7.4) at 37°C . The cells were subsequently superfused with a Ca^{2+} -free solution (solution "A" in which calcium was replaced with $2 \text{mmol}\cdot\text{L}^{-1}$ $MnSO_4$). When the fluorescence intensity (I) was stabilized after a metal (Me^{2+}) treatment (I_{Me}) (usually after 10–15 min), the cardiomyocytes were lysed with 0.1% Triton X-100 (Sigma) to release the dye for recording the background signal, I_{back} . Control experiments were performed on unloaded cells at similar experimental settings. Because I_{Me} and I_{back} represent the fluorescence signals with ionomycin- Me^{2+} before and after lysis, the maximal fluorescence I_{max} from the Ca^{2+} -saturated dye was calculated using the formula $(I_{Mn} - I_{back})/0.2 + I_{back}$. Cytosolic Ca^{2+} was then estimated by the equation $[Ca^{2+}]_i = K_d(I - I_{min})/(I_{max} - I)$, where a value of $1.1 \mu\text{mol}\cdot\text{L}^{-1}$ was assumed for the dissociation equilibrium constant K_d . This value represents a minimal estimate of the K_d of fluo-3 in the cytoplasmic environment (Kao et al. 1989). Because both I_{max} and I_{min} were expressed in terms of I_{Mn} , the only parameters that were determined experimentally were I_{Mn} and I_{back} . Ionomycin- Mn^{2+} -Triton treatment allowed for the construction of the calibration scale for the calcium transient recordings from which average resting and peak $[Ca^{2+}]_i$ were determined.

Induction of mitochondrial energy transition pore formation in isolated perfused hearts: modulation by calcium

Hearts from diabetic and nondiabetic rats were excised and Langendorff-perfused at 37°C and a pressure of 700 mm H_2O with Krebs-Henseleit solution (K-H) containing (in $\text{mmol}\cdot\text{L}^{-1}$) 118.5 NaCl, 25.0 $NaHCO_3$, 1.2 KH_2PO_4 , 1.18 KCl, 1.2 $MgSO_4$, 11.5 glucose, 1.6 $CaCl_2$, gassed with $95\% O_2$ and $5\% CO_2$ (Ravingerová et al. 2001a). After stabilization perfusion for 15 min, the following variations were made: (i) nondiabetic and diabetic control hearts were perfused for a further 15 min with the basic K-H solution (Ca^{2+} $1.6 \text{mmol}\cdot\text{L}^{-1}$); (ii) diabetic and nondiabetic hearts

with elevated offer of Ca^{2+} were those perfused for a further 15 min with K-H solution in which calcium concentration was increased to $2.2 \text{mmol}\cdot\text{L}^{-1}$; (iii) calcium paradox was induced in the diabetic hearts, which were first perfused for 15 min with calcium at $1.6 \text{mmol}\cdot\text{L}^{-1}$, followed by 5 min perfusion with Ca-free medium, and subsequently perfused again for 15 min with $1.6 \text{mmol}\cdot\text{L}^{-1}$ calcium; and (iv) cardiac arrest was performed in diabetic hearts by intracoronary administration of $5 \text{mmol}\cdot\text{L}^{-1}$ $CdCl_2$ applied as a 5 mL bolus immediately at the end of the 15 min perfusion with $2.2 \text{mmol}\cdot\text{L}^{-1}$ Ca^{2+} . After termination of experiments, all hearts were perfusion-fixed and further processed for a specific cytochemical determination of the activity of the octameric mitochondrial isoform of creatine kinase (mCK).

Cytochemical detection of energy transition pores and their quantitative determination

The method of cytochemical detection of energy transition pores is based on the determination of the octameric mCK described by Biermans et al. (1989). It consists of a series of coupled reactions, the last being the reduction of thiocarbonyl nitroblue tetrazolium salt (for more details see Ziegelhoffer-Mihalovicová et al. 1997). After cytochemical detection of mCK activity, tissue probes were dehydrated and embedded in Epon 812. Thin sections of the embedded tissue were examined in a Tesla 500 electron microscope. A stereological method (Baddeley et al. 1986) was used to evaluate the number of energy transition pores, which was then expressed in units of surface density (Ss). The principle of the latter method is based on the application of a testing grid with a high number of cycloids that is superimposed over the electronmicrographs. Ss is expressed as a ratio between the intersections of cycloids with sites with detected mCK and the intersection of cycloids with mitochondrial membranes.

Determination of adenine nucleotide content in myocardial tissue

After fast thoracotomy applied to thiopental-anaesthetized animals, the hearts were removed using the freeze clamp technique (Wollenberger et al. 1960). The frozen tissue was weighed, pulverized in liquid nitrogen, extracted with $10\% HClO_4$ ($10 \text{mL}\cdot\text{g}^{-1}$), and spun down at $5000g$ and 4°C for 10 min. The supernatant was neutralized with $5 \text{mol}\cdot\text{L}^{-1}$ K_2CO_3 . The resulting $KClO_4$ was removed by filtration and the extract was used for analysis of ATP, ADP, and AMP content by means of HPLC (Beckman Gold, Germany) using a Sepharon SGX C18 column ($250 \times 4 \text{mm ID}$, particle size $7 \mu\text{m}$; Tessek, Czech Republic). A Beckman Ultrasphere ODS column ($45 \times 4.6 \text{mm ID}$, $5 \mu\text{m}$) was applied as precolumn. The mobile phase was composed of $0.1 \text{mol}\cdot\text{L}^{-1}$ KH_2PO_4 and $8 \text{mmol}\cdot\text{L}^{-1}$ tetrabutylammonium hydrogen sulfate in 10% methanol, pH 6, flow rate $1 \text{mL}\cdot\text{min}^{-1}$. Sample injection volume was $20 \mu\text{L}$. Analysis was performed at room temperature.

Chemicals

Streptozotocin, ATP, ADP, AMP, glucose, dextran, bovine serum albumin, taurine, Hepes, EDTA, and EGTA, as well as other biochemicals, if not indicated elsewhere, were

from Sigma-Aldrich and were of analytical grade. Most inorganic salts were purchased from Lachema (Czech Republic) and were also of analytical grade.

Statistics

All data were expressed as means \pm SD, unless stated otherwise. Data for normally distributed parameters (according to Shapiro–Wilk's test) were checked on homoscedasticity of variance by *F* test. Transmembrane potential and calcium transient data were checked for intra- and interindividual variabilities separately in the control and diabetic groups with one-way ANOVA with repeated measures. A similar strategy was also applied in the evaluation of data on surface density of the energy transition pores, and 50 or more electronmicrographs were analyzed from each independent preparation.

The effect of acute diabetes on continuously developing changes in biochemical and biophysical variables, such as parameters characterizing the metabolic state, functional parameters of mitochondria, or membrane fluidity, was tested by unpaired Student's *t* test. To test for dual effects (experimental groups or time course of DPH penetration in the evaluation of membrane fluidity) we used one-way ANOVA with repeated measures. Ratiometric data (transmembrane potential, calcium transients, surface density of energy transition pores) were analyzed with the nonparametric Mann–Whitney test. To evaluate differences in the categorical variables (transmembrane potential) the statistical test for 2 independent proportions was applied. The associations between variables were analyzed with Pearson's correlation coefficients from simple linear regression analysis. Our statistical significance reporting criteria for comparisons between data sets with equal and unequal variances were $p < 0.05$ or $p < 0.1$, respectively. All statistical analyses were performed with StatsDirect 2.3.7 software (Cheshire, UK).

Results

Acute streptozotocin diabetes in rats

As early as day 2 after streptozotocin administration, the diabetic rats ($n = 15$) exhibited significant glucosuria amounting to 20–25 mol·L⁻¹. Full development of the disease on day 8 after streptozotocin was confirmed by increases in blood glucose from 5.08 \pm 0.21 to 20.56 \pm 0.95 mmol·L⁻¹ and blood glycohemoglobin from 4.30% \pm 0.14% to 7.25% \pm 1.02% (controls vs. diabetic animals, $n = 15$; $p < 0.01$ each) and by a decrease in the level of serum insulin from 1.09 \pm 0.16 to 0.58 \pm 0.10 pkat·g⁻¹ protein ($n = 8$ each, $p < 0.01$) (1 katal (kat) equals 1 mol·s⁻¹).

Functional parameters of the mitochondria

The functional parameters in mitochondria from acute diabetic rat hearts are present in Table 1. The expected damage was manifested by a significantly decreased state S₃ and state S₄ oxygen consumption (Q_{O_2}). The data in Table 1 show decreases by 21.92% in S₃ and by 14.80% in S₄ with succinate (oxidized via complex II) as well as by 26.08% (S₃) and by 29.24% (S₄) with glutamate + malate (oxidized via complex I). Respective values of the respiratory control index (RCI) in Table 1 also exhibit a decrease by 7.23% with succinate and by 17.84% with glutamate + malate as

substrates. This indicates that the dysfunction in complexes I and II of the respiratory chain may have similar outcomes. We also observed a slowdown in the flow of electrons along the respiratory chain, which became manifested in a significant drop in the oxidative phosphorylation rate (OPR) and resulted in a decrease in the capacity of the mitochondrial energy-generating system. Nevertheless, the data on the ADP:O ratio given in Table 1 indicate that in this phase of the disease, the damages to substrate oxidation and the subsequent depression in the OPR were not yet associated with any significant degree of uncoupling between the processes of oxidation and phosphorylation.

Fluidity of lipid bilayer and content of conjugated dienes in membranes of isolated heart mitochondria

Fluidity of the bulk lipids of the mitochondrial membranes was assessed by DPH. The fluorescent probe incorporates spontaneously into the hydrophobic core of the membranes, and its signal strongly depends on the degree of freedom of the phospholipid acyl chain movements. A decrease in the fluorescence signal (*r*) reflects an increase in the fluidity. Data in Figure 1 reveal that 8 days of diabetes induced an elevation in the fluidity of the membrane lipids of rat heart mitochondria ($p < 0.05$).

Analysis of the data on the content of conjugated dienes in the membrane bilayer of the mitochondria revealed only nonsignificant elevation ($p > 0.05$) in conjugated diene formation in the mitochondria isolated from diabetic hearts in comparison with those present in the mitochondria of nondiabetic control hearts (128.32 \pm 15.71 and 113.60 \pm 14.47 μ mol·g⁻¹ protein, respectively).

Transmembrane potential of isolated heart mitochondria

Estimation of the transmembrane potential in cardiac mitochondria isolated from nondiabetic and 8-day streptozotocin-diabetic rats was performed by using fluorescent dye JC-1 and calculating the ratio of fluorescence intensity of JC-1 aggregates and monomers at excitation 584 nm and emission 531 nm. Fluorescence was collected from 50 and 53 single mitochondria from 10 nondiabetic and 10 diabetic hearts and the ratio amounted to 0.803 \pm 0.110 in the controls and 0.704 \pm 0.108 in the diabetic heart mitochondria. The difference between both transmembrane potentials failed to reach statistical significance, but we observed a trend for a decrease in the transmembrane potential in the diabetic hearts with a probability of 0.053, a value close to the threshold of significance of 5%.

Calcium transients in nondiabetic and diabetic hearts

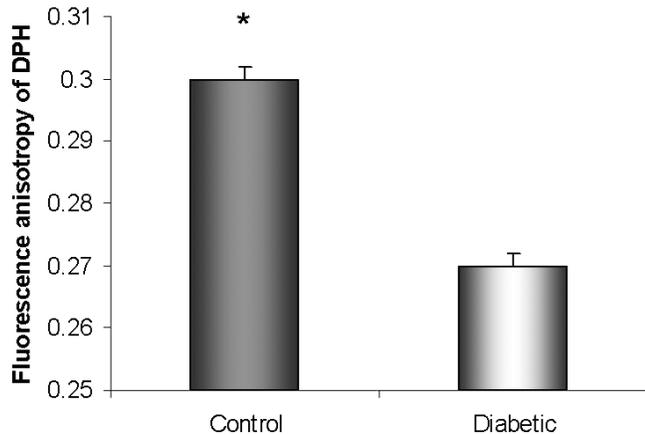
Amplitudes of calcium transients were defined as a ratio of the peak (systolic) and resting (diastolic) [Ca²⁺]_i. Mean level of resting [Ca²⁺]_i in the nondiabetic and diabetic cardiomyocytes amounted to 102 \pm 8 nmol·L⁻¹ and 113 \pm 10 nmol·L⁻¹, respectively. Average peak [Ca²⁺]_i concentrations in the nondiabetic and diabetic cardiomyocytes amounted to 1790 \pm 293 nmol·L⁻¹ and 2080 \pm 305 nmol·L⁻¹, respectively. Results in Fig. 2 represent fluo-3 fluorescence collected from the whole internal area of single cardiomyocytes. The results confirm that the diabetic hearts exhibited significantly augmented calcium transients ($p < 0.05$).

Table 1. Functional parameters of mitochondria from acute diabetic rat hearts.

	Control	Diabetic	Significance
Q_{O_2}			
$Q_{O_2}(S_3)$ -glut / mal	151.31±4.31	111.86±3.93	0.01
$Q_{O_2}(S_4)$ -glut / mal	35.7±1.04	25.26±1.13	0.01
$Q_{O_2}(S_3)$ -suc / mal	238.64±12.14	188.72±8.51	0.01
$Q_{O_2}(S_4)$ -suc / mal	132.33±5.28	112.72±3.93	0.01
RCI			
Glutamate / malate	5.375±0.261	4.415±0.228	0.05
Succinate	1.802±0.039	1.67±0.028	0.05
OPR			
Glutamate / malate	362.66±13.04	262.97±12.97	0.001
Succinate	322.89±12.54	243.46±12.47	0.001
ADP:O			
Glutamate / malate	2.505±0.261	2.405±0.228	NS
Succinate	1.361±0.039	1.31±0.028	NS

Note: Q_{O_2} , oxygen consumption (nanomoles of oxygen atoms per milligram of protein per minute); S_3 and S_4 , state 3 and state 4 respiration; substrates: glut, glutamate; mal, malate; and suc, succinate; RCI, respiratory control index; OPR, oxidative phosphorylation rate (nanomoles of ATP per milligram of protein per minute); ADP:O, adenosine diphosphate : oxygen ratio (molecules of ADP converted to ATP per atom of oxygen); NS, not significant ($p > 0.05$). For details see Materials and Methods.

Fig. 1. Membrane fluidity of isolated rat heart mitochondria estimated by fluorescence anisotropy of DPH in the mitochondrial membrane. Results are means \pm SE from 10 experiments. *, significant at $p < 0.05$ vs. diabetic. For details see Materials and Methods.

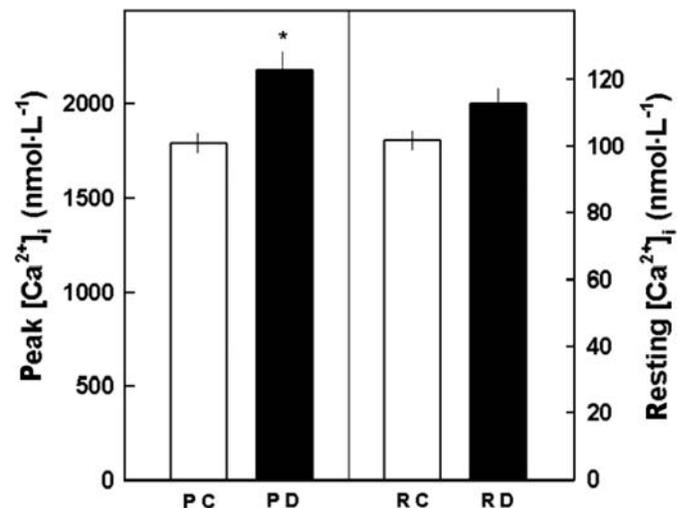


Role of calcium signaling in modulation of energy transition pore formation in rat heart mitochondria

In the nondiabetic control hearts, the elevation of calcium concentration in the perfusate led to an increased calcium influx into the heart cells. This influx consequently augmented the intracellular calcium transients, which in turn propagated a signal, 1st for a rise in cardiac contractility documented by a 30.2% elevation ($p < 0.01$) in the rate of LV contraction (dP/dt) (not shown), which is always coupled with an increase in energy utilization, and 2nd for a significant increase (by 75.5%, $p < 0.01$) in the energy transition pore formation as indicated by Ss elevation (Fig. 3).

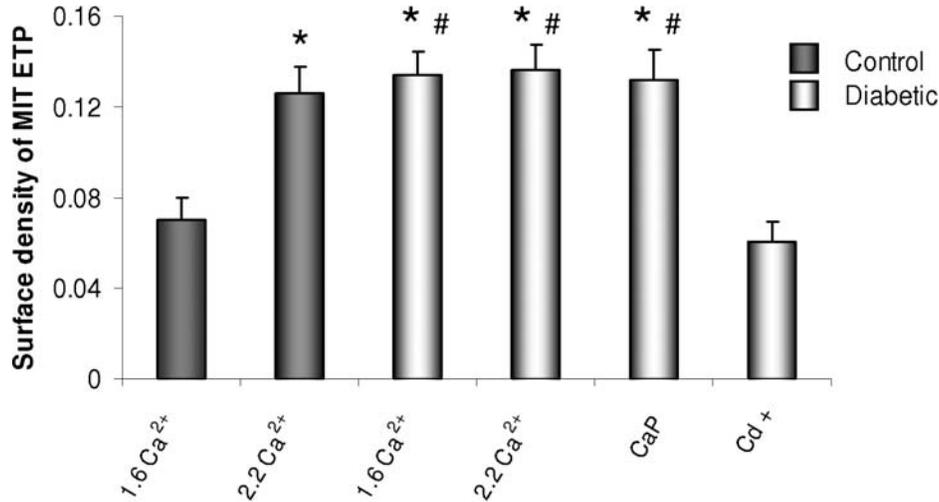
In the diabetic hearts, the basic energy transition pore formation was already identical with that which could be maximally achieved in nondiabetic hearts upon perfusion with elevated calcium. Nevertheless, neither the perfusion with

Fig. 2. Calcium transients in nondiabetic control and diabetic hearts. Average resting $[Ca^{2+}]_i$ in the nondiabetic and diabetic cardiomyocytes was 102 ± 8 nmol·L⁻¹ and 113 ± 10 nmol·L⁻¹, respectively. Average peak $[Ca^{2+}]_i$ concentrations in the nondiabetic and diabetic cardiomyocytes was 1790 ± 293 nmol·L⁻¹ and 2080 ± 305 nmol·L⁻¹, respectively. Results are means \pm SE. *, significant at $p < 0.05$ vs. PC. The mean value of calcium transient amplitude was specified as a ratio of the mean peak (systolic) and mean resting (diastolic) $[Ca^{2+}]_i$ concentrations and was 1688 in controls and 1967 nmol·L⁻¹ in the diabetic cardiomyocytes. For details see Materials and Methods. PC, peak control; PD, peak diabetic; RC, resting control; RD, resting diabetic.



elevated calcium nor the induction of calcium paradox was capable of causing a significant increase in the energy transition pore formation in the diabetic heart mitochondria. This observation indicates that the maximal degree to which the process of energy transition pore formation could be stimulated by calcium signaling had already been achieved. Induction of cardiac arrest by partial replacement of calcium by cadmium ions led to a rapid decay of transition pores and

Fig. 3. Effect of calcium on energy transition pore formation in mitochondria of nondiabetic (light columns) and diabetic (dark columns) rat hearts. Results are means \pm SE from 7 experiments in each group, expressed in units of surface density (Ss) of transition pores in mitochondrial membranes. Groups: Dark columns, nondiabetic control hearts after perfusion with normal calcium ($1.6 \text{ mmol}\cdot\text{L}^{-1}$) or high calcium ($2.2 \text{ mmol}\cdot\text{L}^{-1}$) content in the perfusion medium. First two light columns, diabetic hearts after perfusion with normal ($1.6 \text{ mmol}\cdot\text{L}^{-1}$) or high ($2.2 \text{ mmol}\cdot\text{L}^{-1}$) calcium. Third light column (CaP, calcium paradox), diabetic hearts after perfusion with normal ($1.6 \text{ mmol}\cdot\text{L}^{-1}$) calcium followed by 5 min perfusion with Ca-free medium, and subsequently perfused again with normal calcium for 15 min. Last bright column (Cd+, calcium replacement-induced cardiac arrest), diabetic hearts after perfusion with high ($2.2 \text{ mmol}\cdot\text{L}^{-1}$) calcium immediately followed by intracoronary administration of $5 \text{ mmol}\cdot\text{L}^{-1}$ CdCl_2 applied as a 5 mL bolus. *, significant at $p < 0.01$ vs. 1.6 Ca^{2+} (nondiabetic, normal calcium); #, $p < 0.01$ vs. Cd+ (diabetic, cardiac arrest). For details see Materials and Methods.



thus confirmed that the calcium signal is essential for their formation.

Hypothetic relationship between intracellular calcium signaling, membrane fluidity, and energy transition pore formation in membranes of cardiac mitochondria

Investigation of the coincidence between energy transition pore formation, increase in membrane fluidity, and decrease in transmembrane potential in the heart mitochondria in response to signaling by intracellular calcium transients led us to formulate the following hypothesis (Fig. 4). Via a mechanism not yet determined, the calcium signal carried by the intracellular calcium transient induces an oligomerization of the dimeric mCK molecules present in the space between the inner and outer mitochondrial membranes. The created octameric mCK then binds to the ATP/ADP translocase in the inner mitochondrial membrane and to the porin molecules in the outer mitochondrial membrane. The resulting complex brings both mitochondrial membranes into close contact and creates structures that are termed energy transition pores or contact sites. These mechanically push aside the intermembrane fluid, which then accumulates between the contact sites forming pillow-like structures on the membrane. The stretching in bows of these pillows then increases the free volume between the acyl chains of phospholipids and enables their higher mobility. This is finally observed as an increase in the fluidity of the lipid bilayer of the membrane.

High-energy phosphate content in myocardial tissue

Investigation of the high-energy phosphate content in tissue of the acute diabetic myocardium revealed no significant decrease in the content of ATP and ADP (Fig. 5). A deficit of AMP ($p < 0.05$) proved insufficient to significantly influ-

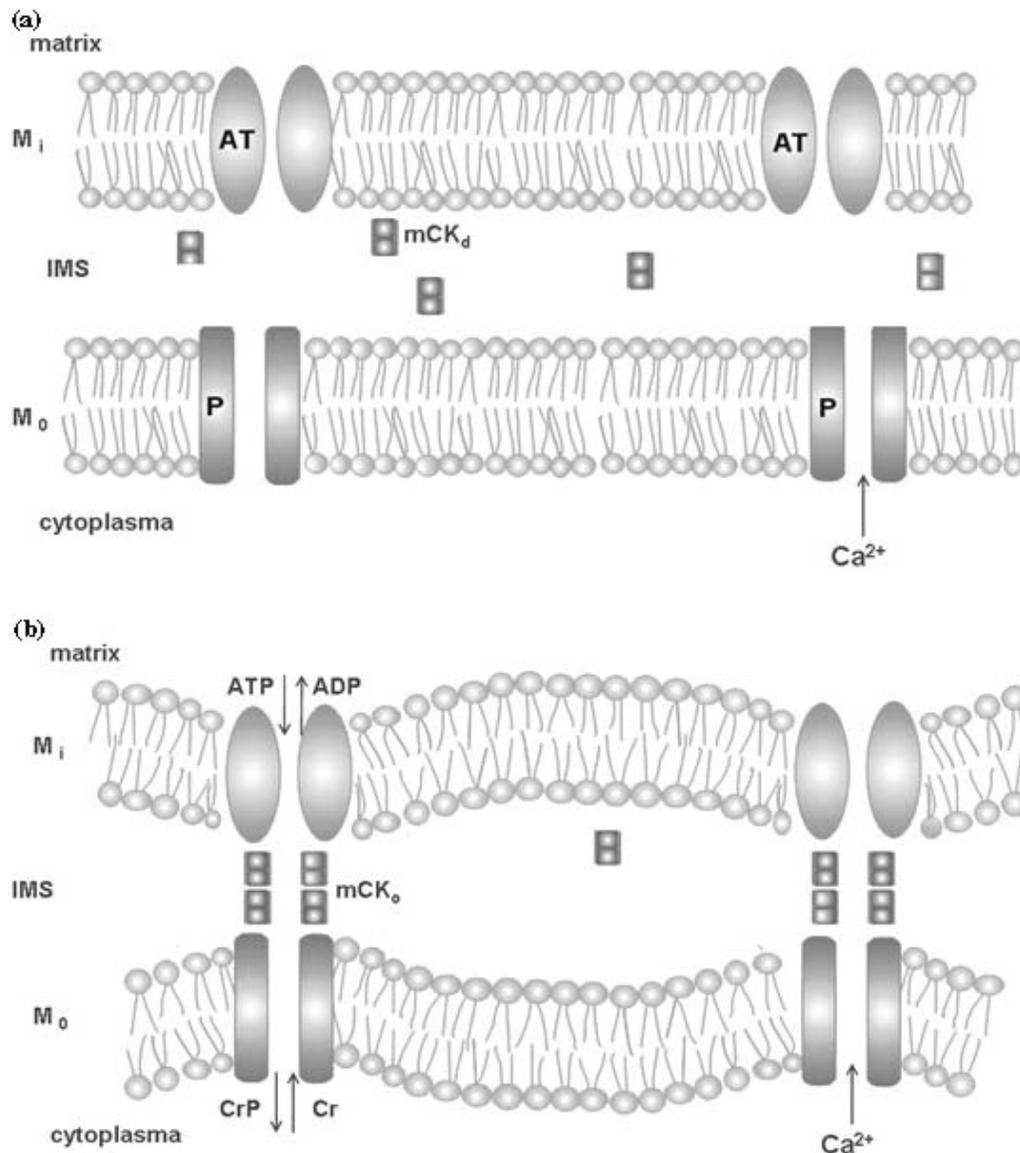
ence the total pool of adenine nucleotides (ΣAN , $p > 0.04$). This finding indicates that in the acute phase of streptozotocin diabetes, a Ca^{2+} -triggered augmentation of energy delivery from the mitochondria to the cytoplasm occurs. Owing to the more abundant energy transition pores in the membranes, heart mitochondria are still more or less capable of meeting the increased energy demands induced in the myocardium by the same increased calcium transients.

Discussion

Previous studies indicated that because of expressed alterations in intracellular calcium handling, streptozotocin diabetes represents an appropriate model for studies on calcium sensitivity of the heart (Kawamura and Suzuki 1991; Feuvray et al. 1991; Tribulová et al. 1996; Ziegelhöffner et al. 1996). This model is particularly relevant to the acute phase of the disease, when the alterations induced directly by diabetes are already strongly manifested, but the disease-accompanying complications are not yet developed (Gøtzsche 1991; Ziegelhöffner et al. 1999; Ferko et al. 2006). Considering the above assumptions, in the present study we utilized the model of 8-day diabetes. Parameters referring to the metabolic state of streptozotocin-injected rats (significant increase in glucose and glycohemoglobin in the blood, decrease of insulin in the serum) presented in the Results of our study confirm that the disease was already fully developed.

The role of malfunctioning mitochondria in cellular pathophysiology was recently reviewed extensively by Seppet et al. (2007). Besides an insufficient production of energy in the form of ATP, jeopardized mitochondria may also produce increased amounts of reactive oxygen species that may lead to alterations in calcium handling by these organelles.

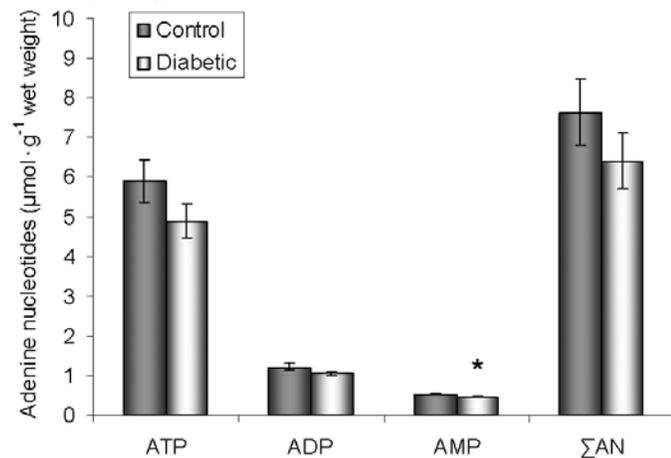
Fig. 4. Role of myocardial isoform of creatine kinase in calcium-triggered formation of energy transition pores in heart mitochondrial membranes. The consequences for the mitochondrial membrane are increased fluidity, possible decreased transmembrane potential, and increased energy delivery to the cardiac cells. Cytoplasm and matrix, cytoplasmic and matrix sides of the mitochondrial membrane; M_i and M_o , inner and outer mitochondrial membranes; P, porin molecules; AT, ATP/ADP translocase; IMS, intermembrane space; Cr, creatine; CrP, creatine phosphate; mCK_d and mCK_o , dimeric and octameric forms of the mitochondrial isoform of creatine kinase. (a) State of the membrane before induction of mitochondrial energy transition pore formation. (b) State of the membrane after induction of mitochondrial energy transition pore formation by Ca^{2+} signal coming from the cytoplasmic side. mCK_d becomes octamerized to mCK_o . Subsequently mCK_o starts docking on the ATP/ADP translocase in the inner mitochondrial membrane and at the same time on the porin in the outer mitochondrial membrane, thus creating a transmembrane structure termed the energy transition pore. For details see Materials and Methods. (From Ziegelh—ffer 2005, p. 202, reproduced with permission of VEDA Publishing House of the Slovak Academy of Sciences, Bratislava, © 2005.)



Functional perturbations of mitochondrial membranes may even initiate or promote apoptosis or necrosis (Seppet et al. 2007). Our results revealed that in the acute diabetic heart, mitochondria experience free radical-induced damage to the respiratory chain (Ferko et al. 2008). This damage led to worsening of the electron transport towards the terminal oxidation and was manifested both by a partial depression in the ability of the mitochondria to utilize oxygen and by de-

creased RCI. In addition, the OPR was also decreased, indicating that despite a still-preserved coupling between the processes of oxidation and phosphorylation, the capacity of the diabetic heart mitochondria to produce ATP declined (Table 1). As a result, the diabetic heart is progressing into a state termed pseudohypoxia, in which the oxygen supply is not restricted, but the heart is unable to utilize it properly. Our previous findings revealed that the diabetes-induced al-

Fig. 5. Levels of adenine nucleotides in cardiac tissue of nondiabetic control and acute diabetic rats. Σ AN, total of the adenine nucleotides. Results are means \pm SE from 8 experiments. *, significant at $p < 0.05$ vs. nondiabetic AMP. For details see Materials and Methods. (From Ferko et al. 2006, reproduced with permission of Gen. Physiol. Biophys., Vol. 25, p. 408,   2006 Institute of Molecular Physiology and Genetics, SAS.)



terations in functional parameters of the mitochondria are associated with changes in fluidity of the lipid layer of the mitochondrial membrane (Ziegelh ffer 2005; Waczul kov et al. 2007; Ferko et al. 2008). This finding supported the assumption that one of the effects of free radicals may be manifested in changes in membrane fluidity.

In our earlier study, heart sarcolemmal membranes from acute diabetic rats exhibited significantly decreased fluidity (Ziegelh ffer et al. 1997), believed to be the result of the action of free radicals, nonenzymatic glycosylation of proteins, and advanced glycosylation product formation. In contrast, at the same experimental conditions the membranes of heart mitochondria showed a significant elevation in membrane fluidity (Fig. 1). This would suggest that in mitochondria either the production of free radicals is lower than assumed, or they are not so vigorously attacking the lipid layer of the mitochondrial membrane. Nevertheless, the formation of free radicals in mitochondria of acute diabetic hearts is well documented. The low lipid oxidation was explained by the hypothesis that the free radicals produced in the respiratory chain (Tretter and Adam-Vizi 2004) react preferably in situ and attack the components of the respiratory chain (Ziegelh ffer 2005; Ferko et al. 2006). To test the validity of this hypothesis, the content of conjugated dienes in membrane lipids was estimated. The results failed to reveal any significant increase in this variable in the acute diabetic heart mitochondria, thus supporting the theory of high affinity of free radicals to the components of the respiratory chain. Nevertheless, it was shown that free radicals may also be produced in the citric acid cycle and in other places in the diabetic heart mitochondria. However, recent findings indicated that the majority of these free radicals are not reacting inside the mitochondria, but are moving to the cytoplasm via the chloride channel (Wenger 2006; Malekov et al. 2007; Holotnakov et al. 2008). All findings mentioned suggest that free radicals modulate mitochondrial membrane fluidity in acute diabetic heart to a lesser extent than would

follow from the scheme presented by Farahmand et al. (2003). However, with the progress of diabetes the metabolic derangement of the heart becomes more intensive (Kuo et al. 1983; Pierce and Dhalla 1984; Rodrigues and McNeill 1992; Ravingerov et al. 1996). The damage caused by radicals and nonenzymatic glycosylation, for example, gradually accumulates, and the extent of damage becomes more in accordance with the Farahmand's scheme (Ziegelh ffer et al. 1996, 1997; Farahmand et al. 2003; Bracken et al. 2003; Neticadan et al. 2003).

Prolonged calcium transients in the heart were reported to occur in each type and in each phase of diabetes. However, data concerning the amplitudes of the calcium transients are more ambiguous (Noda et al. 1993; Kotsanas et al. 2000; Waczul kov et al. 2007), often because of absent calibration of the recordings. In the present study, we have observed only a slight increase in the basic $[Ca^{2+}]_i$ levels in the diabetic cardiomyocytes, in accordance with observations by Xu et al. (1996). However, the peak levels of the calcium transient amplitudes were significantly elevated in comparison with the controls (Fig. 2). These results clearly document the presence of strong calcium signaling already present in the acute phase of the disease.

The calcium signal propagated by calcium transients plays an important role in the endogenous protection of the heart (Ravingerov et al. 2001a, 2001b; Ferko et al. 2006, 2008) including the modulation of energy production and transfer. It was shown that calcium signals are involved in the formation of energy transition pores in mitochondrial membranes (Ziegelh ffer-Mihalovicov et al. 1997, 2002). These pores are ephemeral structures believed to provide increased transfer of energy from the mitochondria to the cytoplasm. Thus the number of mitochondrial energy transition pores may be expected to reflect the changes in energy demands of cardiac cells. In diabetes or under conditions of increased extracellular calcium concentration, calcium-propagated signals considerably amplify the energy demands of cardiomyocytes. So it is reasonable to assume that they may exert a stimulatory effect on mitochondrial energy transition pore formation as well. This is also supported by our results (Fig. 3).

The mechanisms of formation and decay of energy transition pores are not yet fully understood. However, it is known that the maximal density of the energy transition pores is strongly limited by the pool and mobility of their constituting molecules in the mitochondrial membrane. We assume that both the formation and the decay of energy transition pores are rapid processes that may be completed within minutes. Further, all accompanying changes in the structure, transmembrane potential, and fluidity of the mitochondrial membranes are processes that may be running rapidly as well. Consequently, the rapidity of these changes would exclude the possibility of alterations in chemical composition of membrane lipids and almost completely rely on structural changes of the mitochondrial membranes. According to our hypothesis, these structural changes may be mediated via modulation of biophysical properties of the mitochondrial membranes, that is, the changes in the fluidity are triggered directly by energy transition pore formation or decay (Fig. 4). To better understand the membrane fluidity parameter, it is necessary to realize that fluidity is expressed

as the reciprocal value of the fluorescence anisotropy of the probe. Fluorescence anisotropy is related to the degree of lipid ordering or packing density of the acyl chains of phospholipid molecules in the membrane. That means that the less packed the phospholipid acyl chains are, the more the DPH probe becomes immersed into the lipid layer, the less intensive the fluorescence signal is, and consequently, the more fluid the lipid layer of the membrane becomes. According to our hypothesis, in places where the transmembrane pores are created, the intermembrane fluid is mechanically pushed out. This leads to accumulation of intermembrane fluid in the space between the energy transition pores and leads to arching of the membranes, which then resemble pillow-like structures. The mechanical tension in the bows on the membranes directly increases the membrane fluidity by decreasing the packing density between the acyl chains of the lipid layers. In this scenario, the subsequent shift in the transmembrane potential that follows is also a consequence of the described structural changes of the mitochondrial membrane.

The efficacy of increased mitochondrial energy transition pore formation as a means of endogenous protection of energy metabolism was assessed from the actual energy balance in the myocardium in acute diabetes. As shown (Fig. 5), only minor loss occurred in the content of ATP, ADP, AMP, and the total pool of adenine nucleotides. This indicates that in the acute phase of diabetes, the cardiac mitochondria are still able to provide a degree of ATP synthesis and delivery that is sufficient for maintaining the energy balance of the myocardium.

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