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Original article

Studies of the role of tubulin beta II isotype in regulation of mitochondrial respiration in intracellular energetic units in cardiac cells

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ABSTRACT

The aim of this study was to investigate the possible role of tubulin βII, a cytoskeletal protein, in regulation of mitochondrial oxidative phosphorylation and energy fluxes in heart cells. This isotype of tubulin is closely associated with mitochondria and co-expressed with mitochondrial creatine kinase (MtCK). It can be rapidly removed by mild proteolytic treatment of permeabilized cardiomyocytes in the absence of stimulatory effect of cytochrome c, that demonstrating the intactness of the outer mitochondrial membrane. Contrary to isolated mitochondria, in permeabilized cardiomyocytes (*in situ* mitochondria) the addition of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) in the presence of creatine had no effect on the rate of respiration controlled by activated MtCK, showing limited permeability of voltage-dependent anion channel (VDAC) in mitochondrial outer membrane (MOM) for ADP regenerated by MtCK. Under normal conditions, this effect can be considered as one of the most sensitive tests of the intactness of cardiomyocytes and controlled permeability of MOM for adenine nucleotides. However, proteolytic treatment of permeabilized cardiomyocytes with trypsin, by removing mitochondrial βII tubulin, induces high sensitivity of MtCK-regulated respiration to PK–PEP, significantly changes its kinetics and the affinity to exogenous ADP. MtCK coupled to ATP synthasome and to VDAC controlled by tubulin βII provides functional compartmentation of ATP in mitochondria and energy channeling into cytoplasm via phosphotransfer network. Therefore, direct transfer of mitochondrially produced ATP to sites of its utilization is largely avoided under physiological conditions, but may occur in pathology when mitochondria are damaged. This article is part of a Special Issue entitled "Local Signaling in Myocytes".

1. Introduction

Experimental studies of the mechanisms of regulation of mitochondrial function by feedback metabolic signaling *in vivo*[1–15] need the use of the permeabilized cells or fibers technique [16–24] and methods of *in vivo* kinetic studies [4–7]. Intensive investigations during the last two decades with use of these techniques have shown that the regulation of mitochondrial function *in vivo* is very different from that *in vitro*: the apparent Km for exogenous ADP in regulation of respiration is 20–30 times higher in the permeabilized cells than in isolated mitochondria *in vitro*[8–24]. This high apparent Km for ADP can be decreased by addition of creatine that activates mitochondrial creatine kinase, MtCK [8,13,19,20], or by the controlled proteolytic treatment [21–24]. The apparent Km for exogenous ADP shows the availability of ADP for the adenine nucleotide translocase (ANT) in mitochondrial inner membrane (MIM) and was proposed to be dependent on the permeability of the mitochondrial outer membrane's (MOM) voltage-dependent anion channel (VDAC) [22,23]. A strong decrease of the apparent Km for exogenous ADP produced by trypsin treatment pointed to the possible involvement of some cytoskeleton-related protein(s) in the control of the VDAC permeability originally referred to as "factor X" [22,23]. Appaix et al. [24] have shown that among cytoskeletal proteins sensitive to short

Abbreviations: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; ATR, atractyloside; CK, creatine kinase; Cr, creatine; DTT, dithiothreitol; IM, isolation medium; IMS, mitochondrial intermembrane space; MI, Mitochondrial Interactosome; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MtCK, mitochondrial creatine kinase; PCr, phosphocreatine; PBS, phosphate buffer solution; PEP, phosphoenolpyruvate; PK, pyruvate kinase; STI, soybean trypsin inhibitor; VDAC, voltage-dependent anion channel; WS, washing solution.

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proteolytic treatment are tubulin and plectin. Rostovtseva et al. [25,26] established that the first candidate for the role of "factor X" is αβ heterodimeric tubulin, which upon binding to VDAC reconstructed into a planar lipid membrane strongly modulated the channel's conductance. Reconstitution experiments indicated that the addition of the heterodimeric tubulin to isolated mitochondria strongly increased the apparent Km for ADP [27]. Recent immunofluorescence confocal microscopic studies allowed to identify the tubulin associated with mitochondrial outer membrane in cardiomyocytes as its βII isotype [14]. The aim of this study was to investigate further the role of this tubulin-βII isotype in the regulation of respiration in cardiac cells. We show by immunofluorescence confocal microscopy and respirometry that short proteolytic treatment of permeabilized cardiomyocytes removes tubulin-βII from MOM. This significantly increases the MOM permeability for ADP as measured by activation of the MtCK located in the outer surface of inner mitochondrial membrane with trapping of extramitochondrial ADP by the pyruvate kinase (PK) – phosphoenolpyruvate (PEP) system. In accurately prepared permeabilized cardiomyocytes PK–PEP system has no effect on respiration, while in damaged cardiomyocytes and after proteolytic treatment MOM permeability is increased and respiration rate decreased due to ADP tapping by PK–PEP. This permeability test of MOM controlled by tubulin-βII can be used as the most sensitive quality control for intactness of mitochondria in permeabilized cardiomyocytes. Removal of tubulin-βII by proteolytic treatment does not damage the outer mitochondrial membrane itself (as shown by cytochrome c test), but significantly decreases the apparent Km for ADP via an increase of the permeability of VDAC.

2. Materials and methods

2.1. Isolation of cardiac myocytes with perfect rod-like shape, description of various troubleshooting

Adult cardiomyocytes were isolated by adaptation of the technique described previously [19]. Male Wistar rats (300–350 g) were anesthetized and the heart was quickly excised preserving a part of aorta and placed into washing solution (WS) (117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 120 mM sucrose, 10 mM Cr, 20 mM taurine, and 21 mM BES, pH 7.1). All solutions used during the procedure of isolation were saturated with oxygen. The heart was cannulated and washed with WS at a flow rate of 15–20 mL/min for 5 min. At that, the coronary flow should exceed ca. 20 mL/min; otherwise the heart has to be discarded. The collagenase treatment was performed by switching the perfusion to recirculation isolation medium (IM), (117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM creatine (Cr), 20 mM taurine, 10 mM PCr, 2 mM pyruvate, and 21 mM HEPES, pH 7.1), supplemented by collagenase (0.75 mg/mL) at a flow rate of 5 mL/min for 50 min at 37 °C. After the collagenase treatment the system was switched to the initial solution WS for 1–2 min and then the heart was transferred into the IM supplemented with 20 μM $CaCl_2$, 10 μM leupeptin, 2 μM soybean trypsin inhibitor (STI), and 5 mg/mL bovine serum albumin (BSA). The cardiomyocytes were then gently dissociated by pipette suction. The cell suspension was filtered and transferred into a test tube for sedimentation where the calcium-tolerant cells were allowed to freely sediment. After 3–4 min the initial supernatant was discarded and the pellet of cardiomyocytes resuspended in IM containing 20 μM $CaCl_2$, STI and leupeptine. The rod shaped intact cells sedimentated within 2–3 min and the supernatant with damaged cells was discarded. This resuspension–sedimentation cycle with calcium-tolerant cells was performed twice and after that cardiomyocytes were gradually transferred from calcium containing solution into calcium-free Mitomed [17]. Then, the cardiomyocytes were washed 5 times with the Mitomed containing 5 mg/mL BSA, 10 μM leupeptin, and 2 μM STI. Isolated cells were stocked in 1–2 mL volume and stored on ice during further experiments. Isolated cardiomyocytes contained 85–100% of rod-like cells when observed under a light microscope. Final quality of isolated rat cardiomyocytes was found to depend on a number of minor variations in different isolation steps beginning from the severing of the aorta, removal of the heart from the thorax and initial heart perfusion in order to remove Ca^{2+} and the remainder of blood before the collagenase treatment. It is also advisable to perform this operation in ≤1 min to avoid oxygen deficiency and hypoxia. The choice of the collagenase type is the next crucial step; to our experience, collagenase A (Roche) or Liberase Blendzyme 1 (Roche, similar to the new product Liberase DL Research Grade), an artificial mixture of purified enzymes with carefully controlled specific activities (Roche), results in satisfactory results. Caution should be taken in an attempt to reduce duration of the collagenase perfusion time at the expense of the increase in the enzymes activity. For every lot of collagenase the time of dissociation, enzyme ratios, and enzyme concentration affect tissue dissociation outcomes. The perfusion should be performed at controlled rate by pumping and, advisably, under manometric control in order to follow a decrease in the developed pressure from 55 to 60 mm Hg (which corresponds to ≈80 cm H_2O) to that less than 10 mm Hg. Collagenase solution should be washed out in the presence of the mixture of strong inhibitors of serine and thiol proteases and further operations also performed in the presence of these inhibitors. STI is capable of binding to different serine proteases, and leupeptin is the best choice for thiol proteases.

Usually, the obtained preparation is stable enough during 4–5 h needed for measurements. Used saponin concentration and permeabilization time should also be carefully adjusted by studies of the extent of permeabilization by respirometry.

An alternative to isolation of cardiomyocytes is the use of skinned cardiac fibers isolated according to the method described by Kuznetsov et al. and Saks et al. [17,18]. When correctly used, both methods allow obtaining identical results in studies of respiration regulation after cell or fiber permeabilization [8,9,16–24]. In both cases, it is important to avoid artifacts of cell or fiber isolation resulting in misleading and incorrect experimental data, sometimes reported in the literature, when permeabilized cells and fibers have very different properties [28]. The method of preparation of skinned fibers was in details described by Kuznetsov et al.[17]. To isolate high quality cardiomyocytes needed for functional studies it is equally important to avoid multiple errors, which are listed below in the Table 1.

2.2. . Cell preparation for confocal microscopy

Freshly isolated cardiomyocytes and cultured cells were fixed in 4% paraformaldehyde at 37 °C for 15 min. After rinsing with phosphate buffer solution (PBS, containing 2% BSA) cells were permeabilized with 1% Triton X-100 at 25 °C for 30 min. Finally, cells were rinsed repeatedly and incubated with primary antibody as described above for immunoblotting using concentrations indicated in the Table 1 (in 2% BSA containing PBS solution). The next day samples were rinsed and stained for 30 min at room temperature with secondary antibody. Secondary antibodies: Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146), goat polyclonal secondary antibody to mouse IgG-FITC (Abcam ab6785), were used respecting concentrations recommended by the providers (Table 2).

The same proceeding was done during trypsinization of cells but before being fixed, cells were trypsinized by 0.05 or 0.3 μM (0.1–4 mg

Table 1
Useful advises for high quality cardiomyocyte isolation.

Steps	Problems	Possible reasons	Solutions
Heart dissection and hanging to start perfusion	Improper or too high flow rate, see below	Damage of the wall of aorta or aortic valve	Discard this heart. For dissection of the heart holding this between fingers, gently stretch the aorta and cut it to get long aorta to preserve aortic valve from damage
Initial perfusion (80 cm H_2O)	Perfusion pressure too high (>69 mm Hg), coronary flow rate too low (≤15 mL/min)	Aorta partially clogged up	i. Wait for some minutes, small embolus might flow out ii. Remove heart and hang up once again, otherwise discard the heart
	Coronary flow>25 mL/min, abnormally low perfusion pressure	Leak of perfusate due to improper hanging, see above	Hang up the heart once again, otherwise discard the heart
Collagenase perfusion	Perfusion pressure >10–15 mm Hg after 50 min perfusion	Protease concentration too low	Increase the concentration of the collagenase preparation
		Enzyme inactivation	Check storage conditions and the enzyme activity
		Perfusion temperature is too low	Verify temperature
	Too rapid drop perfusion pressure down to zero (in 10–15 min)	Protease concentration too high	Decrease the concentration of the collagenase preparation
	Stained heart surface	Uneven perfusate flow in the heart body due to clogging in some capillaries. Ischemic regions in the heart	Discard the heart, otherwise the yield will be low and quality doubtful
Preparing and washing of the cells	Too low cell sedimentation rate	Substantial amounts of damaged cells present	Normal intact cells sediment in 2–4 min. Elongation of sedimentation time in an attempt to improve the yield could exclusively result in collecting damaged cells
	Low cell viability and yield	Mechanical force for heart dissection is too excessive	Reduce shear force and use the pipette more gently
Saponin treatment	Too low activation of respiration by exogenous ADP	Incomplete permeabilization of sarcolemma	Cell permeabilization has to be checked in the oxygraph cells by addition of the saponin stock solution, the activation of respiration should be complete in ca. 10 min. and the final oxygen consumption rate remain unaltered at least for 20 min, otherwise the saponin concentration should be adjusted
Stirring	Gradual decay in the oxygen consumption rate	Cell damaging due to too vigorous stirring	Decrease in the stirring rate to sufficiently low value

of TR/mg cardiomyocytes protein) for 10 min at 25 °C and then STI, up to a final concentration of 0.02 mM, was added.

2.3. Confocal imaging

The fluorescence images were acquired with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope (Leika, Heidleberg, Germany) equipped with a 63× water immersion objective (HCX PL APO 63.0×1.20 W Corr). Laser excitation was 488 nm for FITC (green fluorescence) and 633 nm for Cy 5 (red fluorescence). Images were then analyzed using Volocity softwere (Improvision, France).

2.4. Colocalization studies

α-actinin and βII-tubulin were immunostained with Cy5-labeled antibody according to the protocol described elsewhere [14]. They were imaged using the 63×/1.4 oil immersion Plan Apo objective, 633 nm HeNe laser and 638–747 nm detection of LSM710NLO confocal microscope (Carl Zeiss). The pinhole value was set to 1 Airy unit. Optical slices closest to the glass surface were analyzed in order to minimize the optical distortions in cardiomyocytes. Mitochondria distribution in fixed cardiomyocytes was visualized using flavoprotein autofluorescence signal excited with the two-photon laser at 720 nm and integrated between 408 and 546 nm. For increasing the autofluorecence of flavoproteins and improving the imaging of mitochondria, the permeabilized cells were treated before fixation with 10 μM rotenone for 10 min under aerobic conditions and washed twice in Mitomed solution described in the next section. The choice of this label-free imaging of mitochondria allowed one to avoid any possible spectral bleed-through to the near-infrared detection channel for α-actinin or βII tubulin immunofluorescence. Indeed, no cell specific background was detected in this channel in unlabelled cardiomyocytes. The very low background signal was detected in case of nonspecific control with the Cy5-labeled secondary antibody. The signal to noise was improved using 16 line scan repetitions and 6 μs pixel dwell time. Overall photobleaching with the used laser intensities did not exceed 1%. The red channel images were not treated for the sake of intensity comparison; the green channel images were processed with a Top-hat square shape filter to improve the contrast of rectangular mitochondria pattern (MetaMorph, Universal Imaging).

Table 2
List of antibodies used.

Commercial name	Dilution for immunofluorescence	Immunogen
Primary antibodies:		
mouse anti-tubulin βII(β2),(Abcam ab28036)	1/1000	Amino acids CEEEEGEDEA at the C terminus
alpha-actinin rabbit (Abcam, ab82247)	1/100	
Secondary antibodies:		
a) Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146)	1/100	
b) goat anti- mouse polyclonal secondary antibody IgG-FITC (Abcam ab6785)	1/800	

2.5. Measurements of oxygen consumption

The rates of oxygen uptake were determined with high-resolution respirometer Oxygraph-2K (OROBOROS Instruments, Austria) in Mitomed solution [17] containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES (pH 7.1), 110 mM sucrose, 0.5 mM dithiothreitol (DTT), 2 mg/mL fatty

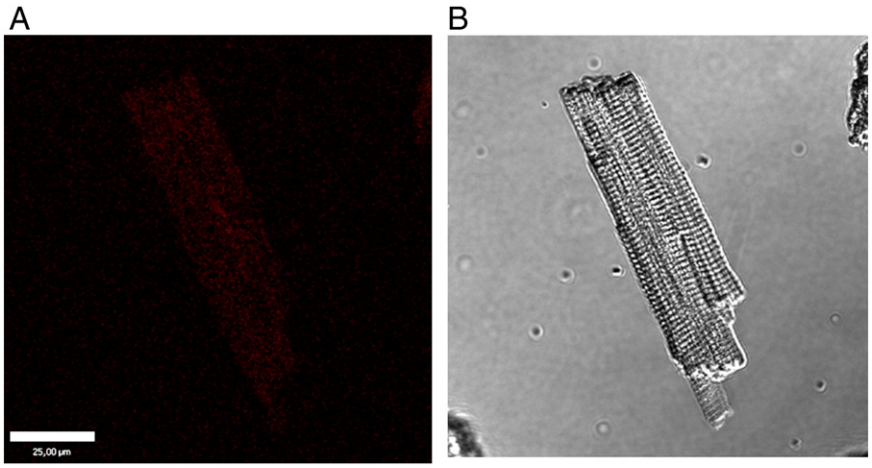

Fig. 1. Specificity test for immunofluorescence labeling of tubulin in cardiomyocytes. A. Confocal image of isolated cardiomyocytes after labeling with secondary antibodies Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch) without primary antibodies. B. Transmission image of the same cardiomyocyte.

acids free BSA, complemented with 5 mM glutamate and 2 mM malate as respiratory substrates. Respiration was activated by addition of creatine to a final concentration of 20 mM in the presence of 2 mM ATP. Maximal respiration rate was measured in the presence of 2 mM ADP. The measurements were carried out at 25 °C; solubility of oxygen was taken as 240 nmoL/mL [17].

2.6. Data analysis

The experiments were carried out independently in two different laboratories. The apparent Km for ADP or ATP was estimated from a linear regression of double-reciprocal plots or by non-linear least-squares.

3. Results

3.1. Confocal immunofluorescence imaging of tubulin-βII in permeabilized cardiomyocytes

Our recent study showed regular localization of βII-tubulin in cardiac cells [14], similar to the "crystal-like" arrangement of mitochondria [29–33]. Therefore, in this work we further investigated the correlation between localization of tubulin-βII close to the outer mitochondrial membrane in adult cardiomyocytes with several important parameters of regulation of mitochondrial respiration in permeabilized cardiac cells *in situ*. The second aim of this study was to describe the necessary quality tests of the intactness of mitochondria in permeabilized cardiomyocytes, required for the proper studies of the interaction of mitochondria with cytoskeleton *in situ*. A short-time proteolysis of permeabilized cells was optimized and used to remove tubulin-βII from the cells, since our earlier studies have shown that tubulin is one of the most sensitive proteins to this kind of treatment [24]. Trypsin treatment is also routinely used for isolation of intact mitochondria from heart muscle [12,24]. The localization of tubulin--βII in fixed cardiac cells was visualized by immunofluorescence confocal microscopy (Figs. 1–4). Fig. 1 shows the high selectivity of this method, demonstrating that incubation of cells with only secondary antibodies does not result in any labeling of intracellular structures. Only after incubation of the fixed and permeabilized cells with primary antibodies against C-terminal tail of tubulin-βII and subsequent incubation with secondary fluorescent antibodies intensive immunofluorescence labeling of tubulin-βII associated with mitochondria can be seen (Figs. 2 and 3). Fig. 2A shows the very regular immunofluorescence labeling of tubulin-βII by using secondary antibodies with green fluorescence before trypsin treatment. Fig. 2B shows that after short proteolysis the fluorescence intensity decreases significantly and regular arrangement of tubulin disappears. Since green fluorescence seen in Fig. 2A and B may be influenced by the autofluorescence of oxidized mitochondrial flavoproteins [18], localization of tubulin-βII was studied also by using secondary antibodies with red fluorescence (Fig. 3). Again, very regular labeling of mitochondria was seen. Similar to the results

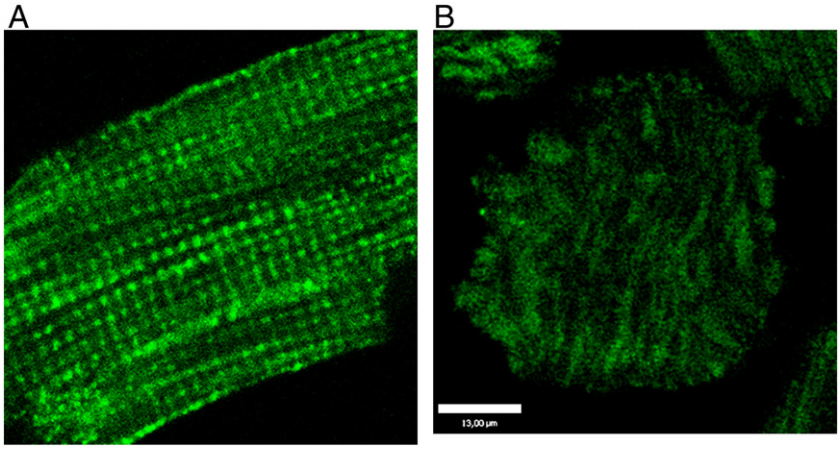

Fig. 2. Immunofluorescence labeling of βII-tubulin before and after trypsin treatment. Labeling with primary antibody and goat anti-mouse polyclonal secondary antibody IgG-FITC (Abcam ab6785). A. Before trypsin treatment. B. After trypsin (0.05 µM) treatment.

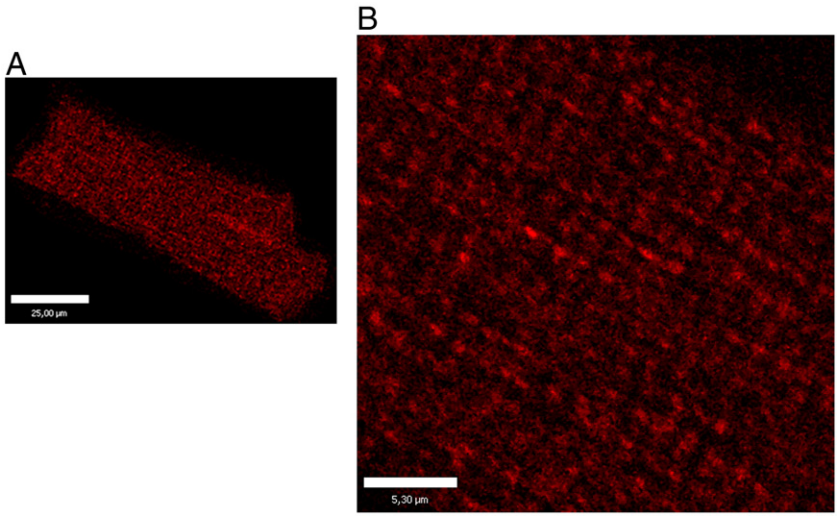

Fig. 3. Immunofluorescence labeling of βII-tubulin in isolated cardiomyocytes with primary and Cy™ 5-conjugated AffiniPure goat secondary anti-mouse IgG (Jackson Immunoresearch). Labeling of mitochondria in parallel rows parallel to long axis of the cell is seen. For further details see Ref. [14].

presented on Figs. 2B, Fig. 4 shows again that short treatment of permeabilized cardiomyocytes with trypsin completely removes the tubulin-βII, also changing the cell shape due to destruction of tubulin and other cytoskeletal systems, and changes intracellular arrangement of mitochondria from regular into irregular clustered one, in accordance with our earlier observations [24,32].

3.2. Colocalization of mitochondria and tubulin βII

To answer the question whether and how tubulin-βII is colocalized with mitochondria in cardiac cells, α-actinin in the Z-lines (Figs. 5A, C) and tubulin-βII (Figs. 5E, G) were immunostained with Cy5-labeled secondary antibody and mitochondrial localization was detected by imaging of the autofluorescence of mitochondrial flavoproteins in oxidized state. Figs. 5A and E show merged images, Figs. 5B, C and F, G show images recorded by separate channels. Figs. 5D and H show the results of quantitative analysis of these images — the fluorescence intensity plots along white lines drawn through representative sequences of 4 mitochondria (see panels B, C and F, G). The very low background signal (dashed lines in Figs. 5D, H) was detected in case of nonspecific control with only the Cy5-labeled secondary antibody. The amplitude of Cy5 fluorescence signal of α-actinin is strongly modulated along a mitochondrial chaplet with the period equal to that of mitochodria but with the inversed phase, indicating essential localisation of α-actinin on Z-lines (Figs. 5A–D). Remarkably, mitochondrial green autofluorescence was not detected in the Z-line area (Fig. 5D), showing the absence of mitochondrial fusion in cardiomyocytes, confirming our previous observation [8]. Contrary to the α-actinin staining, the tubulin-βII fluorescence amplitude modulation is very weak along the line of mitochondrial localization, showing the overall staining of the mitochondria (Figs. 5E–H). Since tubulin βII was detected also in the Z-line area, it seems to form a network-like structures connecting mitochondria to the other cytoskeletal structures. Thus, Figs. 3 and 5 confirm with higher resolution our earlier observations of colocalization of tubulin-βII with mitochondria. However, the resolution limit of confocal microscope (about 0.2 µm) does not allow more detailed analysis of protein localisation on the submitochondrial level (which can be done in the future by using FRET approach).

3.3. Alteration of parameters of respiratory regulation after removal of tubulin βII

The common tests of mitochondrial intactness, which include activation of mitochondrial respiration by ADP, are the cytochrome c test of intactness of the outer membrane of mitochondria and

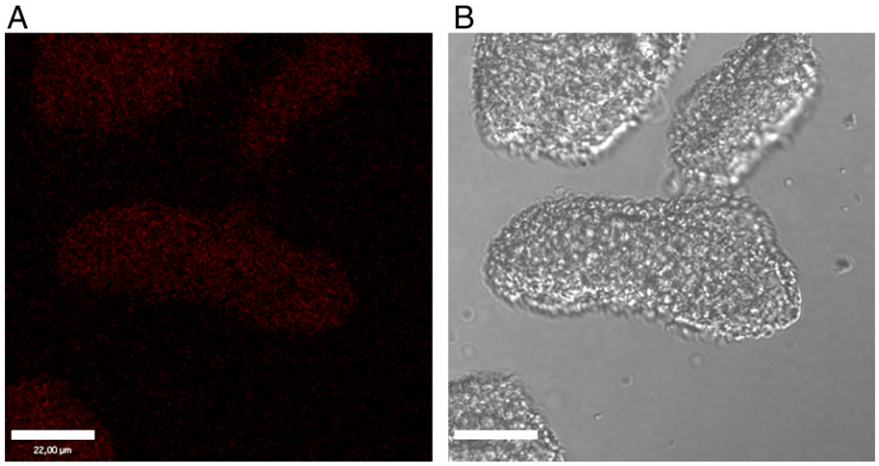

Fig. 4. Immunofluorescence labeling of βII-tubulin after short proteolysis of permeabilized cardiomyocytes with 0.05 µM trypsin before fixation (see Materials and methods). Cy™ 5-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoresearch 115-175-146) was used. A. Confocal image. B. Transmission image of the same cells.

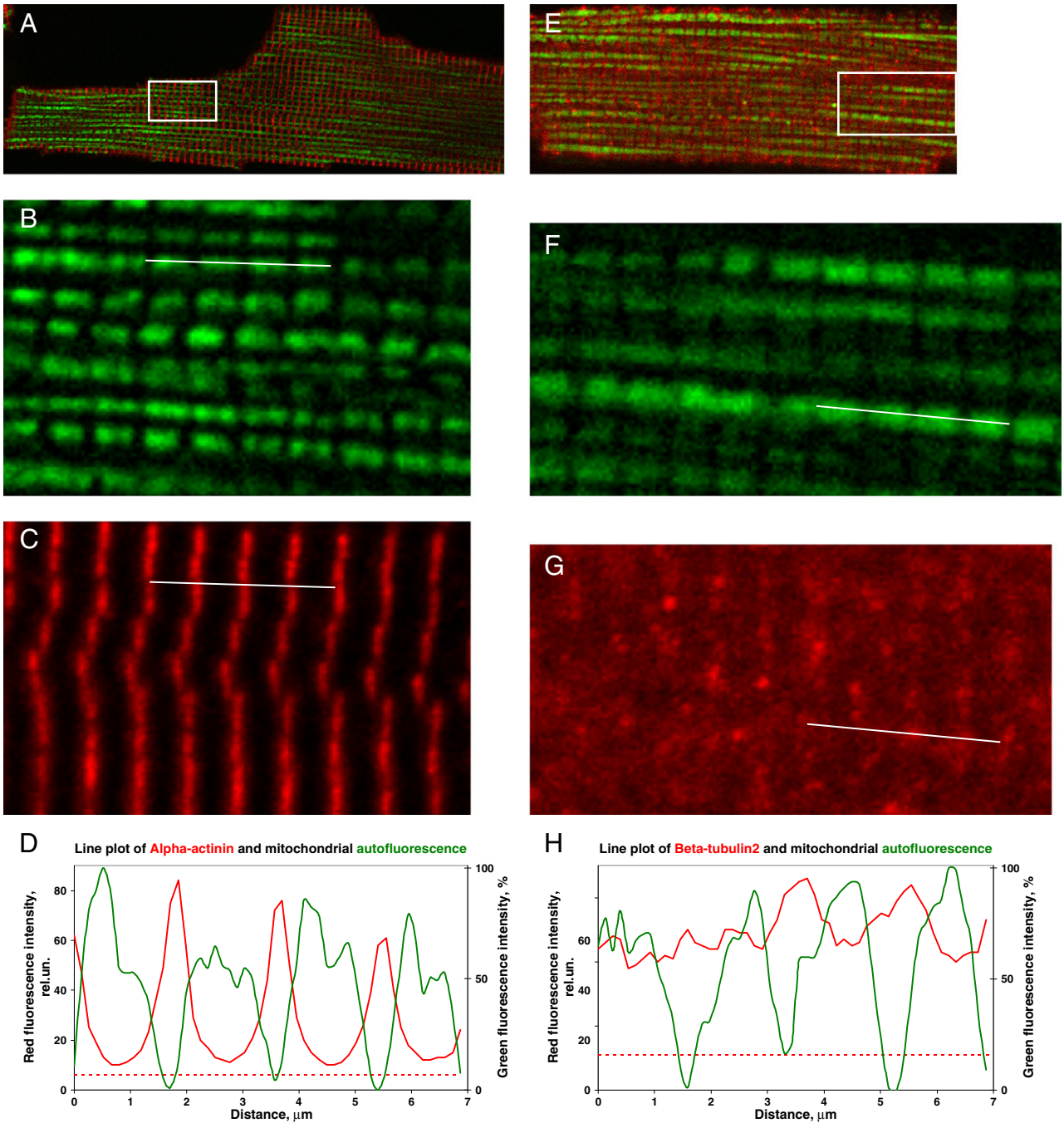

Fig. 5. Comparison of the intracellular distribution of βII-tubulin, α-actinine, a « Z-line label », and mitochondria. Confocal merged images of immunofluorescence labeling of α-actinin (A) or βII-tubulin (E), red color and mitochondrial autofluorescence, green color. B, C ,F, G — zoom in regions of interest highlighted by the white rectangles in the panels A and E, separated channels. D, H — intensity plots along white lines drawn through representative sequences of 4 mitochondria (panels B, C, F, G).Dashed red lines indicate the background level of unspecific fluorescence staining measured in control experiments. Red plots are presented in relative units using the same scale for βII-tubulin and α-actinin. Green plots were normalized to the 100% of the maximal intensity of autofluorescence after the background subtraction.

inhibition of ADP-stimulated respiration by atractyloside (ATR); they are shown in Fig. 6. In permeabilized cardiomyocytes ADP (2 mM) increases respiration rate more than 10 times and this rate is not changed by addition of cytochrome c (Fig. 6A). Cytochrome c, a highly soluble hemoprotein of the respiratory chain is loosely associated with the outer side of the inner membrane of the mitochondria. If the outer membrane is disrupted, cytochrome c leaves mitochondria, and in this situation addition of the protein increases respiration rate [34]. Thus, the cytochrome c test (Fig. 6A) shows that in permeabilized cardiomyocytes mitochondrial outer membrane is entirely intact. ATR completely inhibits ADP-activated respiration, showing that all ADP is imported into mitochondrial matrix *via* ANT [35]. Remarkably, all these parameters are not changed after treatment of permeabilized cardiomyocytes by trypsin (Fig. 6B) that showing that short proteolytic treatment leaves mitochondrial membranes completely intact, in accordance with all earlier data of studies of isolated heart mitochondria [24,34].

More sensitive test which shows clear changes in parameters of regulation of mitochondrial respiration after removal of βII-tubulin by short proteolysis is shown in Fig. 7. This Figure shows the parameters

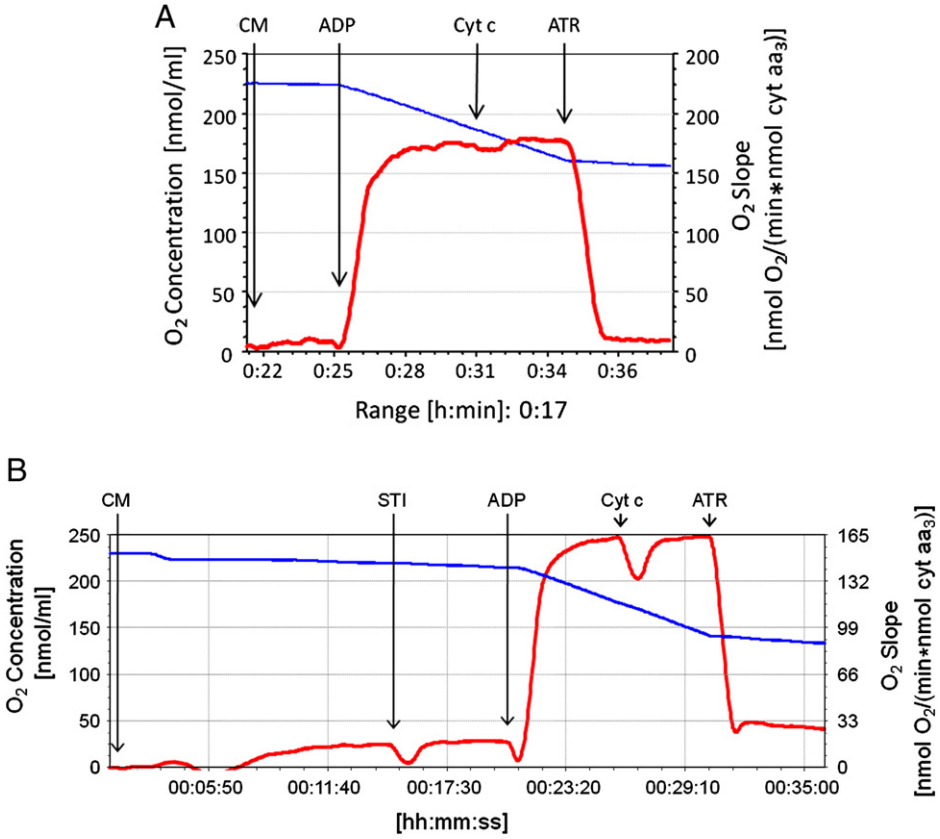

Fig. 6. Representative respiration traces before trypsin treatment of permeabilized cardiomyocytes (CM). A. Respiration is activated with 2 mM exogenous ADP. Cytochrome c (Cyt c) test (10 μM cytochrome) shows the intactness of MOM. Atractyloside (ATR, 0.1 mM) test shows that respiration is totally controlled by ANT. B. The same as A, but after treatment with trypsin at 0.05 μM for 10 min, then soybean trypsin inhibitor (STI) was added.

of regulation of mitochondrial respiration by MtCK activated by addition of creatine and MgATP. MtCK is located at the outer surface of mitochondrial inner membrane in close vicinity of ANT [2,3,36,37] and produces MgADP behind the outer mitochondrial membrane (Fig. 7A). This ADP formed in the active site of MtCK is released into intermembrane space of mitochondria and may either return to matrix *via* ANT or leave mitochondria through VDAC [38,39], the flux distribution between these two routes depending on the permeability of this channel for adenine nucleotides. The ADP flux distribution can be easily revealed by addition of exogenous ADP trapping system consisting of PK (20 U/mL) and PEP (5 mM) (Fig. 7B). Fig. 7C shows that in intact permeabilized cardiomyocytes (more than 90% of rod-like cells) addition of PK–PEP system does not change the rate of respiration, which is maintained at the maximal value by activated MtCK within mitochondrial interactosome. However after short proteolysis, removing βII-tubulin from MOM, addition of PK–PEP system decreases the respiration rate to half of its maximal value (Fig. 7D), as observed for isolated mitochondria before [8,12,40]. That means that about 50% of MgADP produced by MtCK can leave now mitochondria *via* VDAC which permeability for MgADP is increased. Remarkably, the effect of PK–PEP system on the respiration was also seen when the preparation of isolated cardiomyocytes contained, without use of trypsin, about 50% of rod-like intact cardiomyocytes and 50% of round-shape cells, probably due to some damaging factors listed in Table 1 (Fig. 7E). Thus, the PK–PEP test is an important quality control which has to be used in such studies to demonstrate intactness of isolated cardiomyocytes (see in details in Materials and methods section).

Fig. 8 shows that removal of βII-tubulin from mitochondrial membrane decreases the apparent Km for exogenous ADP in regulation of mitochondrial of mitochondrial respiration. This is in good agreement with earlier observation of Kuznetsov et al. and Appaix [21,24]. The results shown in Figs. 7 and 8 support the assumption that βII-tubulin bound to MOM in intact permeabilized cardiomyocytes *in vivo* limits the permeability of VDAC channel and increases ADP transfer to matrix *via* ANT, further enhancing the functional coupling between ANT and MtCK [5,7] and thus increases the functional compartmentation of adenine nucleotides within mitochondria in the cells (Fig. 7B). Under these conditions, the MtCK reaction completely controls the respiration rate even in the presence of cytoplasmic ADP trapping system: increase in creatine concentration rapidly increases the respiration rate to its maximal value (Figs. 9A, C). Under these conditions oxidative phosphorylation is maintained by ADP regeneration and recycling within mitochondrial interactosome coupled to permanent creatine phosphorylation and phosphocreatine production with high PCr/O_2 ratio equal to about 6 [41]. When the βII-tubulin is removed from MOM by proteolytic treatment and the VDAC permeability increased, exchange of adenine nucleotides between mitochondria and medium is increased and MtCK only partially controls the respiration (Figs. 9B, D).

4. Discussion

The results of this work are consistent with an assumption that βII-tubulin is one of the cytoskeletal proteins in heart cells which are able to control selectively the VDAC permeability in mitochondrial outer membrane for adenine nucleotides [14]. This restricted permeability for ADP and ATP favors their recycling in the coupled MtCK–ATP synthasome reactions in mitochondria connecting oxidative phosphorylation to PCr synthesis within a supercomplex, which we called “Mitochondrial Interactosome” [8,41], a key structure of phosphocreatine pathway of intracellular energy transfer [1–15]. Also, limited permeability of VDAC for ADP

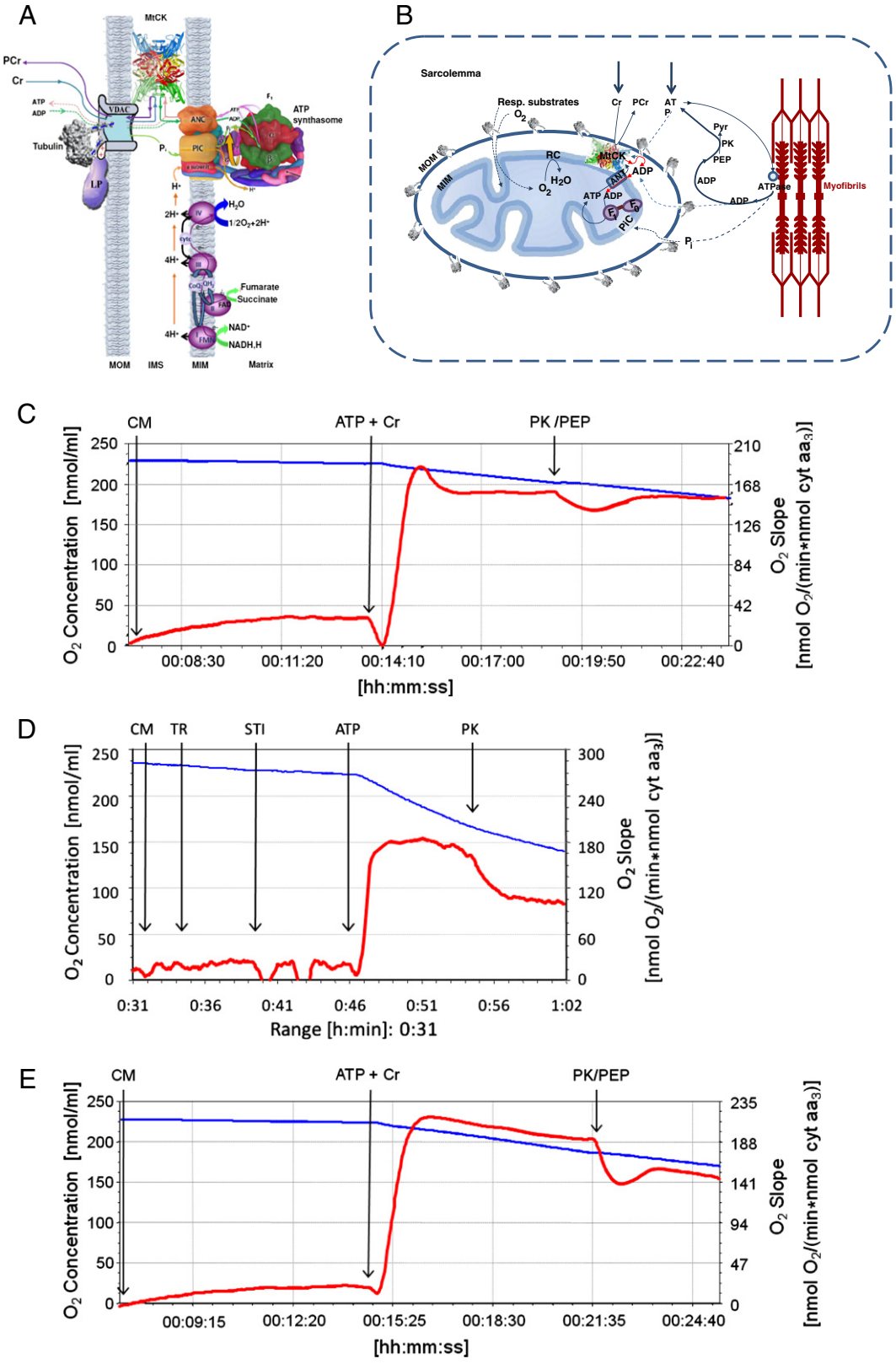

Fig. 7. A. The structure of mitochondrial interactosome showing the localization of MtCK coupled to ATP synthasome in cardiac cells. Adapted from [8,41]. B. The principle of the protocol of the study of ADP fluxes in permeabilized cells by PK–PEP system. C. Respiration of permeabilized cardiomyocytes (CM) was activated by MgATP (2 mM) and creatine (Cr, 20 mM). No effect of the addition of PK–PEP system on the respiration is seen, showing that the MgADP produced by MtCK is not accessible for this system. D. Respiration of trypsin (TR) treated cardiomyocytes in the presence of 20 mM Cr and 2 mM ATP. The oxygen uptake expressed in nmoL O_2/(min∗nmol cyt aa_3). Proteolytic treatment inhibited by addition of soybean trypsin inhibitor (STI, 0.02 mM) and BSA (5 mg/mL). Even after treatment of isolated cardiomyocytes with very low trypsin concentration (0.05 μM) ADP becomes accessible to the PEP–PK trapping system (PK 20 U/mL, PEP 5 mM). E. The effect of PK–PEP system on respiration of permeabilized cardiomyocytes which contained only about 50% of rod-like intact cells.

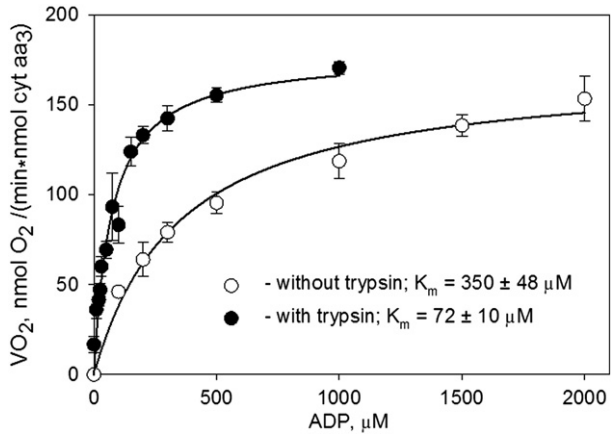

Fig. 8. Effect of trypsin on apparent Km for ADP of mitochondrial respiration in isolated permeabilized cardiomyocytes: respiration rates without (○) and with (●) trypsin treatment. Proteolytic treatment with trypsin resulted in an increase in the affinity of respiration for free ADP due to the proteolytic removal of βII-tubulin. Mean values and standard deviations for 9 experiments are shown.

has an important physiological function preventing from rapid saturation of ANT by this substrate and thus making possible the feedback metabolic regulation of mitochondrial respiration during workload changes [4,7,8,11,13]. Revealing the nature of interaction of tubulins with VDAC needs however further studies by using more selective methods than proteolysis.

Two decades ago two important observations were made almost simultaneously in the studies of cardiac cell bioenergetics. Using electron microscopy, Saetersdal et al. [42] have demonstrated in 1990 the presence of the immunogold anti-β-tubulin labeling at the outer mitochondrial membrane in cardiomyocytes, as well as in myofibers in close opposition to this membrane. This observation rested almost unnoticed and its importance unexplained for these two decades. In parallel, first Kummel in 1988 [16] and then many other investigators in different laboratories ([17–24], reviewed in Ref. [8,9]) discovered the differences in mitochondrial behavior *in vitro* and in permeabilized cardiomyocytes *in situ*: apparent Km for exogenous ADP in regulation of mitochondrial respiration was shown to be 20–30 times higher in the latter case than in isolated mitochondria [8,9]. Detailed investigation of this phenomenon in our laboratories led to conclusion that this phenomenon is related to the tight interactions between mitochondria and cytoskeleton in cardiac cells [22,23]. It was proposed that some components of cytoskeleton may control the permeability of the VDAC channel in the outer mitochondrial membrane in cardiac cells *in vivo*[22,23]. The results of the present and several other recent investigations confirm this suggestion and demonstrate directly that there is a specific isotype of tubulin-βII which is attached to the outer mitochondrial membrane and controls its permeability [14]. Mitochondrial βII-tubulin is co-expressed with MtCK and together with ATP Synthasome they were assumed to form a Mitochondrial Interactosome (MI), a key structure of the phosphotransfer pathway of energy transport into cytoplasm [14]. Evidently, this shows the important role of mitochondrial tubulin, discovered by Saetersdal et al. [42] in 1990.

Nevertheless, many questions still remain unanswered. Tubulin in non-polymerized form exists as αβ-heterodimer [43–45], and there are several isotypes of both subunits which differ mostly by the structure of C-terminal tail [43]. The questions that remain unanswered are: 1) why only βII-tubulin is associated with mitochondria; 2) which is the α isotype; 3) how they both interact with VDAC; and 4) what kind of other cytoskeletal proteins may be involved.

In this work we describe also the very simple and effective tests for investigation of the intactness of MI structure and function, energy fluxes from mitochondria into cytoplasm and functioning of MI which can be used as important quality controls for preparations of cardiac cells or myocardial fibers. Among other methods the cytochrome c test (Fig. 6) is first of them to be used for the detection of intactness of MOM in isolated mitochondria as well as in skinned fibers and permeabilized cardiomyocytes [17,18,34]. The loss of relatively weekly bound cytochrome c from MIM (as an important component of respiratory chain), especially at elevated ionic strength is accompanied by a significant decrease of the oxygen consumption and ATP synthesis [34]. Addition of saturating amount of exogenous cytochrome c to cytochrome c depleted mitochondria in cells or fibers in respiration medium results in restoration of the oxygen consumption and ATP synthesis from exogenous ADP, thus enabling to estimate the degree of damage and an amount of mitochondria with disrupted MOM. However, this effect does not allow estimating the state and quality of MI intactness, functioning and regulation of ATP/PCr synthesis. Inhibition of ANT by CAT [17,35] is another useful tool to check intactness of MIM, since increased rate in the residual oxygen consumption after inhibition by CAT is indicative for bypass of ADP–ATP and thus damage of MIM.

Some indication of functionally coupled MtCK could be observed from the creatine effect on the cellular respiration under conditions of externally added ADP, where creatine added to the experimental medium switches on the MtCK activity, resulting in a substantial decrease in Km(ADP) from values >300 μM down to 80–100 μM due to recycling of ADP in intermembrane space [19,24].

The use of the PK/PEP system is the most sensitive and comprehensive test for intactness of the whole MI system including the regulations at MOM. This simple and effective competitive enzyme method for studying the functional coupling phenomenon, namely the pathway of ADP movement from MtCK back to mitochondria or into the medium, was developed by Gellerich et al. and Guzun et al. [12,13,40,41]. These authors used an external PEP–PK system to trap ADP and thus to compete with ANT for this substrate. This competitive enzyme system was able to suppress 50% of Cr-stimulated respiration in isolated heart mitochondria, thereby showing the rather effective channeling of ADP from MtCK to the medium [12,40]. However, in permeabilized cardiomyocytes when MI is activated with 20 mMCr, PK/PEP system does not have any access to the intramitochondrial ADP and it is not affecting oxidative phosphorylation inside mitochondria and respiration rate. This protocol is excellent to elucidate the role of the mitochondrial outer membrane in the control of MI function, and foresaw many important functional aspects of the control of mitochondrial function *in vivo*. All these tests show that there is practically no measurable direct flux of ATP from mitochondria when MI is actively functioning. Direct transfer of ATP is observed under pathological conditions when the MOM is broken or tubulin lost from MI.

Any disturbances in MOM permeability regulation, including mild protease treatment, result in leakage of ADP and its competing trapping by the excessive PK/PEP and, finally, in a remarkable decrease of respiration. Only in the case of high cell quality (more than 95% intact rod like cells) cell respiration shows absence of the PK/PEP system effect.

The normal shape of the cardiac cells and mitochondrial arrangement are maintained by cytoskeletal structures, including tubulins, plectin, desmin and others [46–58]. In normal adult saponin-skinned fibers intermyofibrillar mitochondria retain their crystal-like pattern along with a relatively slow fluctuations around their position [32,33]. It has been supposed [33] that these fluctuations reflect the configurational changes of mitochondrial matrix between two classical condensed and orthodox

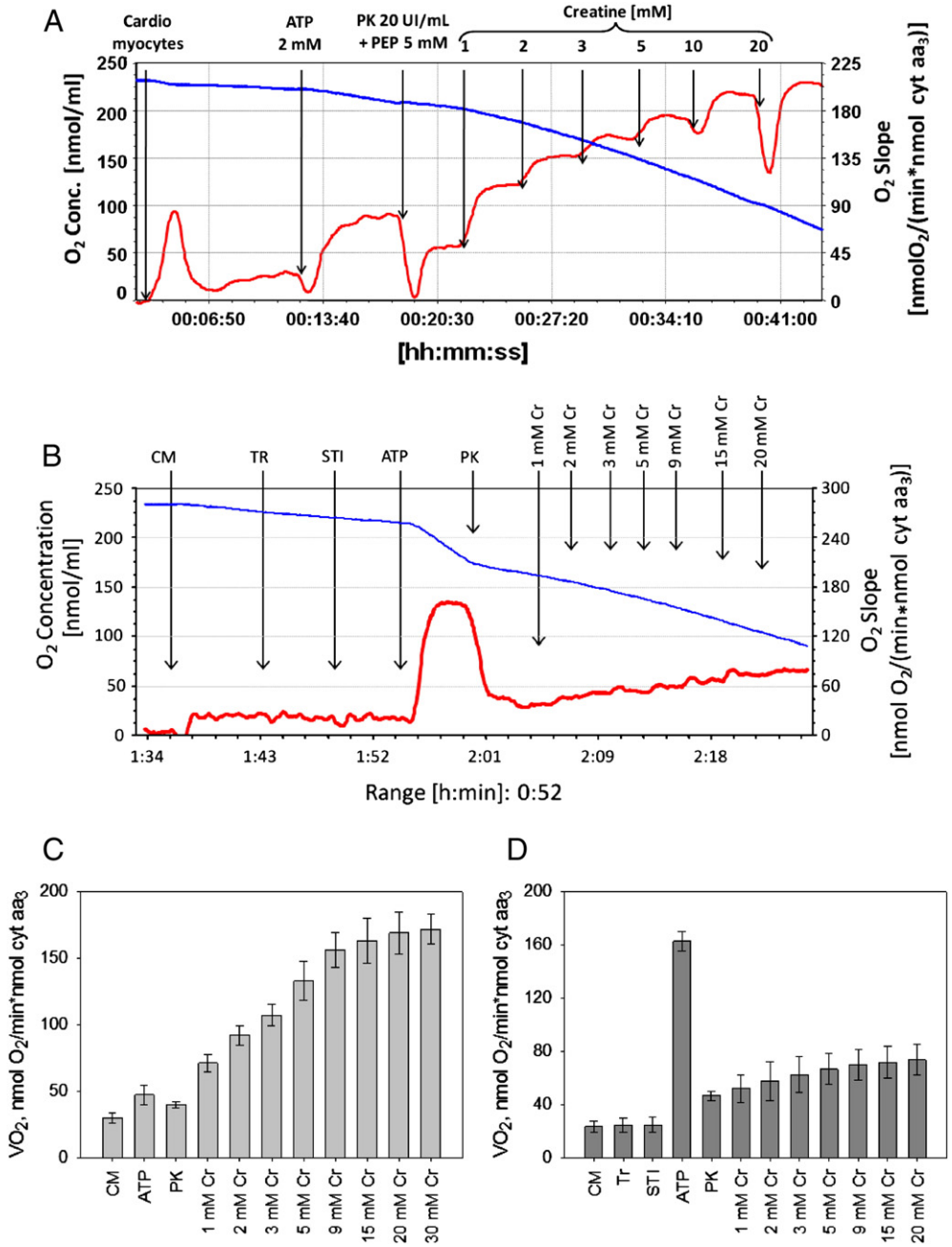

Fig. 9. Regulation of mitochondrial respiration by creatine (Cr) in the presence of activated MtCK, in cardiomyocytes (CM) non-treated and treated with trypsin. A. Cardiomyocytes were permeabilized by saponin and State 2 respiration recorded. MgATP was added to 5 mM final concentration to stimulate MgATPases, this increasing the respiration rate. This rate was decreased by addition of PK (20 U/mL) in the presence of PEP (5 mM). Subsequent addition of creatine rapidly increased the respiration rate up to maximal value. ADP produced by MtCK is not accessible for the PK–PEP system and is rapidly taken up by ANT into mitochondrial matrix. B. The same protocol after trypsin treatment: extramitochondrial ADP is more accessible to the PEP–PK reaction due to the proteolytic treatment, which destroys cytoskeletal proteins involved to the regulation of MOM. C. Mean values and standard errors for 7 experiments described in panel A. D. Mean values and standard errors for 7 experiments described in panel B.

states. Mitochondrial fusion and fission were not seen in adult intact cardiomyocytes [33]. This conclusion is confirmed by the results shown in Fig. 5D in this study.

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Disclosure statement

None.

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