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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

Marcela Gonzalez-Granillo [a], Alexei Grichine [b], Rita Guzun [a], Yves Usson [b], Kersti Tepp [c], Vladimir Chekulayev [c], Igor Shevchuk [c], Minna Karu-Varikmaa [c], Andrey V. Kuznetsov [d], Michael Grimm [d], Valdur Saks [a,c,*], Tuuli Kaambre [c]

[a] INSERM U1055, Laboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, Grenoble, France
[b] Life science imaging —in vitro platform, IAB CRI U823 Inserm/Joseph Fourier University, France
[c] Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia
[d] Cardiac Surgery Research Laboratory, Department of Heart Surgery, Innsbruck Medical University, Innsbruck, Austria

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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.

* Corresponding author at: Laboratory of Bioenergetics, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn, Estonia. Tel.: +372 33 4 76 63 56 27; fax: +372 33 4 76 51 42 18.
E-mail address: valdur.saks@ujf-grenoble.fr (V. Saks).

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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