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# Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria

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**The origin of significant differences between the apparent affinities of heart mitochondrial respiration for exogenous ADP in isolated mitochondria *in vitro* and in permeabilized cardiomyocytes or skinned fibres *in situ* is critically analysed. All experimental data demonstrate the importance of structural factors of intracellular arrangement of mitochondria into functional complexes with myofibrils and sarcoplasmic reticulum in oxidative muscle cells and the control of outer mitochondrial membrane permeability. It has been shown that the high apparent $K_m$ for exogenous ADP (250–350 $\mu$M) in permeabilized cells and in ghost cells (without myosin) and fibres (diameter 15–20 $\mu$m) is independent of intrinsic MgATPase activity. However, the $K_m$ may be decreased significantly by a selective proteolytic treatment, which also destroys the regular arrangement of mitochondria between sarcomeres and increases the accessibility of endogenous ADP to the exogenous pyruvate kinase–phosphoenolpyruvate system. The confocal microscopy was used to study the changes in intracellular distribution of mitochondria and localization of cytoskeletal proteins, such as desmin, tubulin and plectin in permeabilized cardiac cells during short proteolytic treatment. The results show the rapid collapse of microtubular and plectin networks but not of desmin localization under these conditions. These results point to the participation of cytoskeletal proteins in the intracellular organization and control of mitochondrial function in the cells *in vivo*, where mitochondria are incorporated into functional complexes with sarcomeres and sarcoplasmic reticulum.** *Experimental Physiology* (2003) **88.1**, 175–190.

It is a well-known phenomenon that in permeabilized oxidative muscle cells the apparent $K_m$ for exogenous ADP in the control of mitochondrial respiration is very high, in the range of 250–350 $\mu$M, in contrast with isolated mitochondria *in vitro* (where the apparent $K_m$ for ADP is 15–20 $\mu$M: Kummel 1988; Saks *et al.* 1991, 1993, 1994, 1995, 1998*b*, 2001; Fontaine *et al.* 1995; Veksler *et al.* 1995; Kuznetsov *et al.* 1996; Kay *et al.* 1997*a,b,c*; Milner *et al.* 2000; Anflous *et al.* 2001; Braun *et al.* 2001; Liobikas *et al.* 2001; Seppet *et al.* 2001; Toleikis *et al.* 2001; Boudina *et al.* 2002; Burelle & Hochachka, 2002; Dos Santos *et al.* 2002). Simplistic explanation of these differences by the formation of ADP concentration gradients between the medium and the core of the cells can be excluded, since the Brownian movement of ADP in the water solution is much more rapid for a diffusion distance of less than 10 $\mu$m than the metabolic turnover of ADP and ATP (Saks *et al.* 2001). A high value of the apparent $K_m$ for exogenous ADP is also observed in 'ghost' muscle cells after the extraction of myosin in 800 mM KCl (Kay *et al.* 1997*b*). Moreover, we know that the rupture of outer mitochondrial membrane in hyposmotic conditions decreases the value of this parameter (Saks *et al.* 1993). Thus, in the cells *in vivo* the low permeability of the outer mitochondrial membrane for ADP and ATP is controlled by some intracellular factors. The decrease of apparent $K_m$ for exogenous ADP



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during a short, selective treatment of permeabilized cells with trypsin (Kuznetsov *et al.* 1996) points to the participation of some cytoplasmic proteins, most probably related to the cytoskeleton, in the intracellular mitochondrial organization and control of the permeability of the mitochondrial outer membrane (Saks *et al.* 1995). Therefore, the purpose of this study was to use confocal microscopy to investigate the changes in cytoskeletal structures and intracellular mitochondrial distribution in the permeabilized cardiac cells after a 5–15 min period of proteolytic treatment. The results showed rapid collapse of tubulin and plectin networks but not of desmin localization under these conditions, confirming the possibility of participation of these cytoskeleton-associated proteins in the control of mitochondrial function in the cells *in vivo*.

## METHODS

### Animals

Wistar rats were used in all experiments. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

### Isolation of mitochondria from cardiac muscle

Mitochondria were isolated from rat hearts as described by Saks *et al.* (1975).

### Isolation and culturing of adult cardiac myocytes

Calcium-tolerant myocytes were isolated by perfusion with a collagenase-containing medium as described earlier by Kay *et al.* (1997*b*).

### Preparation of skinned muscle fibres

Skinned (permeabilized) fibres were prepared from rat cardiac muscle according to the method described earlier (Saks *et al.* 1998*b*).

### Determination of the rate of mitochondrial respiration in skinned fibres and cardiomyocytes

The rates of oxygen uptake were recorded by using a two-channel high resolution respirometer (Oroboros oxygraph, Paar KG, Graz, Austria) or a Yellow Spring Instruments oxygraph in solution B or sucrose solution, containing respiratory substrates (see below) and 2 mg $ml^{-1}$ of bovine serum albumin (BSA). Determinations were carried out at 25 °C and the solubility of oxygen was taken as 215 nmol $ml^{-1}$ (Kuznetsov *et al.* 1996).

### Confocal microscopy

**(1) Simultaneous imaging of mitochondrial flavoproteins and NADH.** Isolated saponin-permeabilized cardiomyocytes or fibres were attached in a Heraeus flexiperm chamber (Hanau, Germany) with a microscopic glass slide. Then 200 $\mu$l of respiration medium were immediately added into the chamber and equilibrated with air.

The autofluorescence of mitochondrial flavoproteins and NADH was imaged using a confocal microscope (LSM510 NLO, Zeiss) with a × 40 water immersion lens (NA 1.2). The use of water immersion prevented geometrical aberations when observing living cells *in vitro*. The autofluorescence of flavoproteins was excited with the 488 nm line of an argon laser, the laser output power being set to an average of 8 mW. The fluorescence was collected through a 510 nm dichroic beam-splitter and a 505–550 nm band-pass filter. The pinhole aperture was set to one Airy disk unit.

The autofluorescence of NADH was imaged by two-photon excitation using a femto-second pulsed infra-red laser (Tsunami+MilleniaVIII, SpectraPhysics, Darmstadt, Germany). The pulse frequency was set at 100 MHz with a pulse width of 100 fs. The infra-red line was tuned to 720 nm. The laser output power was set to 400 mW. The fluorescence signals were collected through a multiline beam splitter with maximum reflections at 488 ± 10 nm (for rejection of the 488 nm line) and above 700 nm (for rejection of infra-red excitation). A second 490 nm beam splitter was used to discriminate the NADH signal from the flavoprotein signal. Then the flavoprotein signal passed through a 500–550 nm band-pass filter, with an additional infra-red rejection filter before being collected through a pinhole (one Airy disk unit). The NADH signal was redirected to a 390–465 nm band-pass filter with an additional infra-red rejection filter.

A state of fully oxidized mitochondrial flavoproteins was achieved by substrate deprivation.

A maximal NAD(P)H signal was obtained in the presence of substrates.

**(2) Imaging of mitochondria by tetramethylrodamine ethyl ester (TMRE).** To analyse mitochondrial distribution and mitochondrial inner membrane potential, myocytes or fibres were incubated for 30 min at room temperature with 50 nM TMRE, added to respiration medium B. In control experiments dissipation of membrane potential was observed after addition of 5 $\mu$M antimycin A, 4 $\mu$M FCCP and 0.5 $\mu$M rotenone (data not shown). The fluorescence of TMRE in loaded myocytes or fibres was excited with a 543 nm helium–neon laser. The laser output power was set to 1 mW. For co-localization studies of mitochondria and mitochondrial redox potential analysis, the autofluorescence of flavoproteins was excited with the 488 nm line of an Argon laser, the laser output power being set to an average of 8 mW. The fluorescence signals were collected through a multi-line beam splitter with maximum reflections at 488 ± 10 nm (for rejection of the 488 nm line) and at 543 nm (for rejection of the 543 nm line). A second 545 nm beam splitter was used to discriminate the TMRE signal from the flavoprotein signal. Then the flavoproteins signal passed through a 505 nm long-pass filter before being collected through a pinhole (one Airy disk unit). The TMRE signal was redirected to a 560 nm long-pass filter before being collected through a pinhole (one Airy disk unit).

**(3) Labelling of mitochondria with MitoTracker Green FM.** In some cases the mitochondrial imaging was performed by labelling mitochondria with MitoTracker Green FM (excitation 490 nm–emission 516 nm, Interchim), 100 nM, for 45 min at 4 °C in solution B with 2 % of bovine serum albumin (Sigma). This fluorescent probe is non-fluorescent in aqueous solutions and fluorescent only in the lipid environment of mitochondria; it appears to preferentially accumulate in mitochondria regardless of mitochondrial membrane potential, is well retained after fixation with paraformaldehyde and is an important tool for determining the mitochondrial mass. Cardiomyocytes or fibres were then washed with phosphate buffer solution (PBS) containing (mM): NaCl 56, $KH_2PO_4$ 1.5, KCl 2.7 and $Na_2HPO_4$ 8 (Biomedia, Boussens, France). The labelled cells were deposited on glass coverslips and mounted in a mixture of Mowiol (Calbiochem, La Jolla, USA) and glycerol to which 1,4-diaza-bicyclo-[2,2,2]-octane (Acros Organics, Pittsburgh, PA, USA) was added to delay photobleaching. Samples were observed by confocal microscopy (LSM510 NLO, Zeiss) with a × 40 oil immersion, NA 1.4, objective lens (see above).

**Table 1. Apparent $K_m$ values ($\mu$M) for exogenous ADP in the regulation of mitochondrial respiration in the media without and with $K^+$**

| Preparation | Sucrose medium without $K^+$ | Solution B with $K^+$ | References |
|---|---|---|---|
| Isolated mitochondria | 12 ± 3.5 | 19 ± 7 | Saks *et al.* 1991; Liobikas *et al.* 2001; Dos Santos *et al.* 2002 |
| Permeabilized cardiac cells or fibres | 306 ± 16 | 320 ± 26 | Saks *et al.* 2001; Boudina *et al.* 2002 Toleikis *et al.* 2001; etc. (see text) |

**(4) Double labelling of mitochondria (MitoTracker) and cytoskeletal proteins (immunofluorescence).** Double labelling of cytoskeletic proteins and mitochondria was performed on permeabilized fibres or cardiomyocytes in suspension. Cells were first incubated with 100 nM MitoTracker Green FM (Interchim) for 45 min at 4 °C in solution B with 2 % bovine serum albumin (Sigma). They were then washed in solution B before being fixed with 4 % paraformaldehyde for 20 min at room temperature for desmin and plectin antibodies or with methanol for 5 min at –20 °C for tubulin antibody. Cardiomyocytes or fibres were washed with PBS containing (mM): NaCl 56, $KH_2PO_4$ 1.5, KCl 2.7 and $Na_2HPO_4$ 8, (Biomedia) and incubated in PBS/BSA 2 % with primary antibodies of cytoskeletal proteins overnight at 4 °C. Either monoclonal anti-$\beta$-tubulin (mouse IgG1 isotype) antibody (Sigma) at 1/200, or polyclonal anti-desmin (rabbit) antibody 1/200 (gift of L. Rappaport, INSERM Unit 127, Paris), or monoclonal anti-plectin (mouse IgGI isotype) antibody 1/200 (Sigma) were used. Monoclonal anti-$\beta$-tubulin was immunospecific for tubulin, as determined by indirect immunofluorescence staining and immunoblotting procedures; monoclonal anti-plectin was immunospecific for plectin by the same criteria: it recognizes an epitope located in the middle section of the rod domain of the plectin molecule. After two or three washes in PBS, cells were incubated for 3 h in PBS/BSA 2 % with secondary antibody rhodamine (TRITC)-conjugated AffiniPure $F(ab')_2$ Fragment Donkey Anti-Mouse IgG (excitation 503 nm–emission 530 nm) at 1/50 (Interchim). This time, cardiomyocytes or fibres were washed in PBS and 3 times in distilled water. The labelled cells were deposited on glass coverships and mounted in a mixture of Mowiol and glycerol to which 1,4-diazabicyclo-[2,2,2]-octane (Acros Organics) was added to delay photobleaching. Samples were observed by confocal microscopy (LSM510 NLO, Zeiss) with a ×40 oil immersion, NA 1.4, objective lens.

### Determination of the pyruvate kinase activity

The activity of pyruvate kinase (PK) in stock solutions was assessed by a coupled lactate dehydrogenase system. The decrease in the NADH level was determined spectrophotometrically in a Uvikon 941 (Kontron Instruments, UK) in solution B supplemented with 0.3 mM NADH, 2 mM ADP, 1 mM phosphoenolpyruvate (PEP) and 4–5 i.u. $ml^{-1}$ lactate dehydrogenase in response to the addition of different amounts of PK.

### Protein concentration determination

Protein concentration in mitochondrial preparations was determined by the ELISA method using the $EL_x$ 800 Universal Microplate Reader from Bio-Tek Instruments and a BCA kit (protein assay reagent) from Pierce (USA).

**Solution A.** Composition (mM): $CaK_2EGTA$ 1.9, $K_2EGTA$ 8.1, $MgCl_2$ 9.5, dithiothreitol (DTT) 0.5, potassium 2-(*N*-morpholino)-ethanesulfonate (K-Mes) 53.3, imidazole 20, taurine 20, $Na_2ATP$ 2.5, phosphocreatine 15, pH 7.1 adjusted at 25 °C.

**Solution B.** Composition (mM): $CaK_2EGTA$ 1.9, $K_2EGTA$ 8.1, $MgCl_2$ 4.0, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, $K_2HPO_4$ 3, and in oxygraphy experiments also glutamate 5 + malate 2, pH 7.1 adjusted at 25 °C.

**Sucrose solution.** Composition (mM): sucrose 240, K-Mes 20, EGTA 0.5, glutamate 5, malate 2, $K_2HPO_4$ 3, $MgCl_2$ 5, pH 7.1 adjusted at 25 °C; 2 mg $ml^{-1}$ of BSA was added.

**Reagents.** All reagents were purchased from Sigma (USA) except ATP and ADP, which were obtained from Boehringer (Germany).

### Analysis of the experimental results

The values in Table 1 and the figures are expressed as means ± S.D. The apparent $K_m$ for ADP was estimated from a linear regression of double-reciprocal plots.

## RESULTS AND DISCUSSION

### Regulation of mitochondrial respiration by ADP in isolated mitochondria *versus* mitochondria *in situ*: some new illustrations

Figure 1 shows typical recordings of changes in oxygen consumption by isolated rat heart mitochondria *in vitro* in response to successive additions of different amounts of ADP. These experiments were performed in solution B with a $K^+$ concentration of 126 mM, and the respiratory substrates present were glutamate and malate. In accordance with classical respiratory control phenomenon (Chance & Williams, 1956), small amounts of ADP were consumed rapidly in state 3 and the respiration rate decreased to that of state 4 rate because of the lack of ADP and the very low rate of its regeneration by a small amount of $Mg^{2+}$-ATPase contamination in the purified mitochondrial preparation (Fig. 1*A*). Addition of the hexokinase and glucose as an ADP-regenerating system allowed ADP levels to be maintained and corresponding respiration rates to be constantly in state 3. It was also possible to record precisely the kinetics of the regulation of respiration by ADP in this state (Fig. 1*B*). Maximal state 3 respiration rate was achieved after the addition of 90–100 $\mu$M ADP, and the apparent $K_m$ for ADP was 19 ± 7 $\mu$M in these types of experiments (Table 1), in good agreement with many earlier observations (Saks *et al.* 1985, 1991; Liobikas *et al.* 2001; Dos Santos *et al.* 2002). This apparent affinity constant is very close to that of the adenine nucleotide translocator (Vignais, 1976) due to the high permeability of the outer mitochondrial membrane for ADP and ATP in mitochondria *in vitro* (Klingenberg, 1970). The kinetics of the regulation of respiration was not changed by the

5–15 min treatment of isolated mitochondria with trypsin (results not shown). Proteases are used for the pretreatment of muscle tissue before the isolation of mitochondria, to avoid mechanical damage to these structures. The necessity of this pretreatment will be explained by the results described below.

It was found in 1988 (Kummel, 1988), and then reproduced later in numerous studies, that the kinetics of the regulation of respiration by ADP observed in mitochondria *in situ* either in permeabilized cardiomyocytes or in carefully prepared permeabilized muscle fibres are significantly different to those observed *in vitro* in isolated mitochondria (Kummel, 1988; Saks *et al.* 1991, 1993, 1994, 1995, 1998*b*, 2001; Veksler *et al.* 1995; Fontaine *et al.* 1995; Kuznetsov *et al.* 1996; Kay *et al.* 1997*a,b,c*; Milner *et al.* 2000; Anflous *et al.* 2001; Braun *et al.* 2001; Liobikas *et al.* 2001; Seppet *et al.* 2001; Toleikis *et al.* 2001; Boudina *et al.* 2002; Burelle & Hochachka, 2002; Dos Santos *et al.* 2002). Figure 2 shows the confocal microscopic images of mitochondria in three types of preparations used to study mitochondria regulation *in situ*: isolated and permeabilized cardiomyocytes (Fig. 2*A* and *B*), permeabilized (skinned) fibres (Fig. 2*C*) and ghost fibres obtained after extraction of myosin, which, however, does not change the regular arrangement of mitochondria in the cells (Fig. 2*D*). In all cases the diameter of fibres is close to that of isolated cardiomyocytes, due to careful separation of fibres from each other (Saks *et al.* 1998*b*). Therefore, the ADP diffusion distance from the medium into the core of the cell does not exceed 10 $\mu$m. In all these preparations the kinetics of regulation of respiration by exogenous ADP are similar (Fig. 3), and very different from that for isolated mitochondria *in vitro* (Fig. 1). In Fig. 3*A* it can be seen that much higher concentrations of ADP are needed to fully activate the state 3 respiration in permeabilized cardiac fibres than for the activation of respiration of isolated mitochondrial *in vitro*. Figure 3*B* shows the linear regression of double-reciprocal plots of ADP kinetics for isolated permeabilized cardiomyocytes or fibres, and respectively, ghost cells or fibres. All these preparations

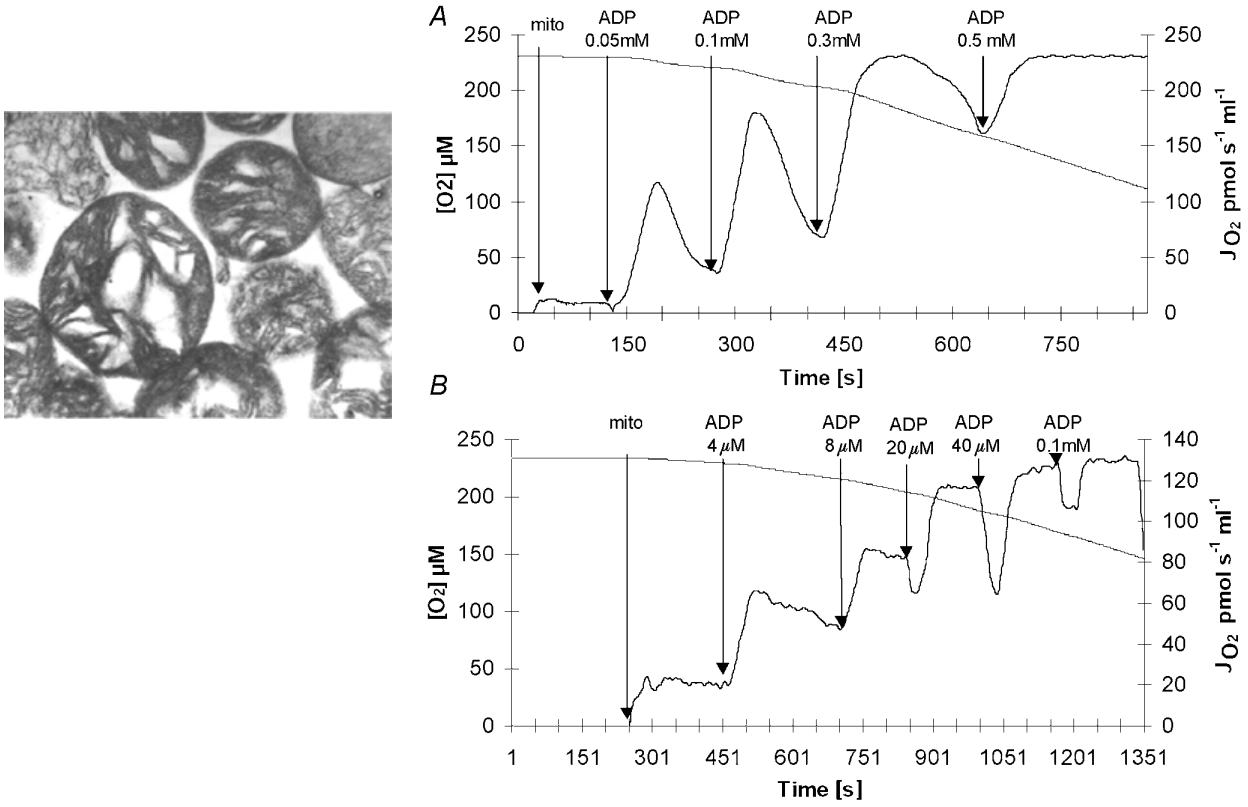


**Figure 1**

Kinetics of regulation of respiration by ADP in isolated rat heart mitrochondria *in vitro*. The graphs show two recordings: the change of oxygen concentration with time (left ordinate axis) and its first derivation (right ordinate axis) that shows directly the rate of oxygen consumption. *A*, isolated heart mitochondria without ADP-regenerating system. After every addition of ADP it is consumed rapidly in state 3 and the respiration rate decreases to state 4. *B*, the presence of glucose and hexokinase (an ADP-regenerating system) in the medium enables ADP levels to be maintained and respiration to be constantly in state 3; the apparent $K_m$ can be determined precisely. The apparent $K_m$ for isolated mitochondria is 10–20 $\mu$M. Inset to the left shows electron microscopy of isolated heart mitochondria.

give a family of superimposed straight lines in double-reciprocal plots, and for all of them the apparent $K_m$ for exogenous ADP is in the range 250–350 $\mu$M without creatine in the respiratory medium. This means that the phenomenon observed is of an intracellular nature, and that in the permeabilized fibre preparations the cells are well separated (as evidenced in Fig. 2), thus avoiding any further problems for the diffusion of exogenous ADP into these fibres. Because of the presence of intracellular $Mg^{2+}$, $Ca^{2+}$-ATPases, the ADP is regenerated continuously maintaining constant steady-state rate of respiration (Fig. 3*A*), and addition of the exogenous ADP-regenerating hexokinase–glucose system does not change the respiratory regulation kinetics (Seppet *et al.* 2001).

In all preparations described above the addition of creatine exerts a strong effect on the apparent $K_m$ (ADP) (Fig. 3*B*). From a physiological point of view, this is the most important mechanism of the control of respiration by the coupled mitochondrial creatine kinase reaction in oxidative muscle cells: it increases the turnover of adenine nucleotides severalfold by the regeneration of ADP in the mitochondrial intermembrane space, maintaining a high rate of aerobic phosphocreatine production and metabolic stability in the cells (Wallimann *et al.* 1992; Kay *et al.* 2000).

Figure 4 shows an important property of these permeabilized cell and fibre preparations: that the apparent $K_m$ for exogenous ADP is independent of the intrinsic $Mg^{2+}$-ATPase activity of fibres. In ghost cardiac fibres from which myosin has been removed by treatment with 800 mM KCl, cell shape is well preserved and a very regular arrangement of mitochondria can be seen forming parallel layers with a clear striation pattern, resembling the

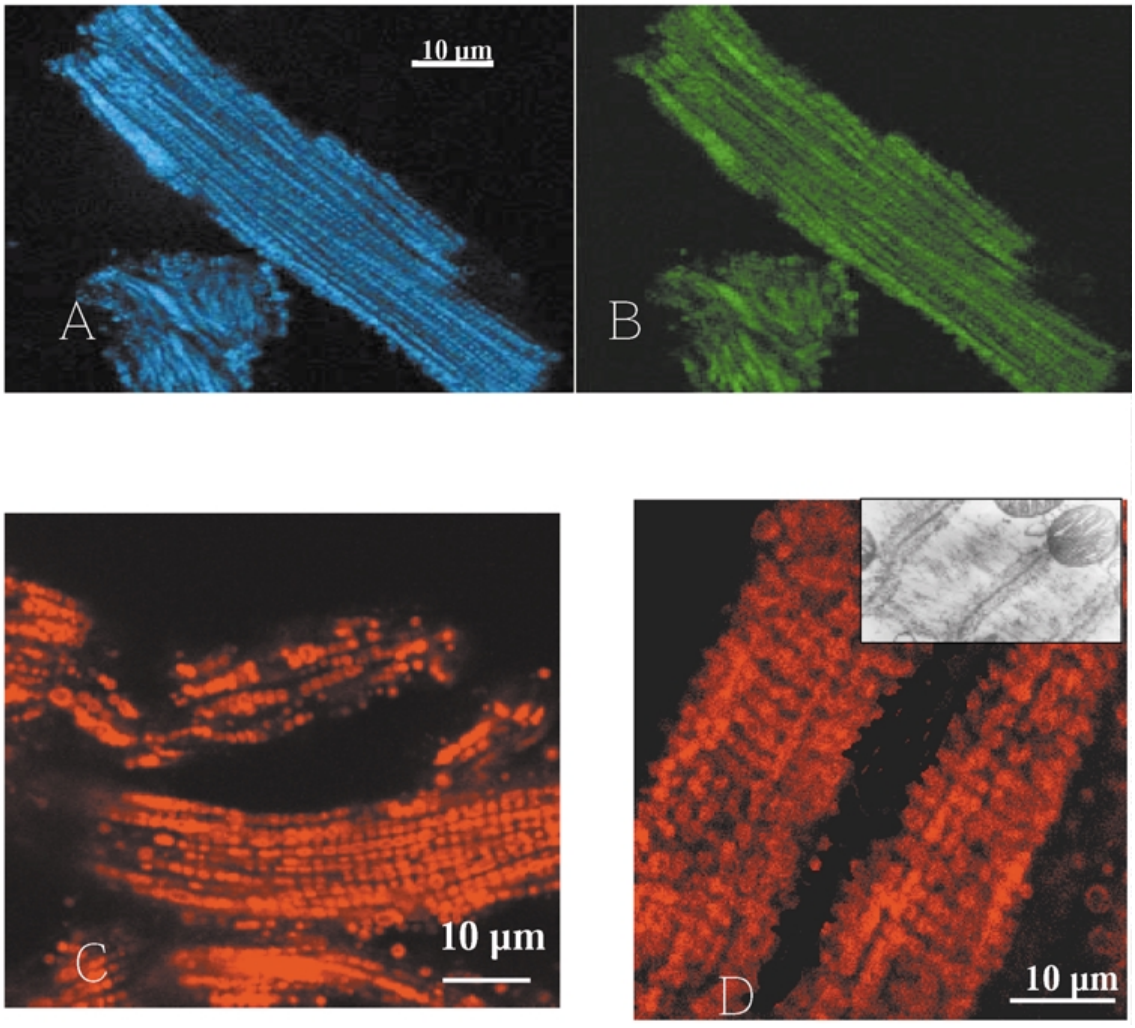


**Figure 2**

Confocal imaging of mitochondria by NADH autofluorescence observed in the presence of substrate (glucose, *A*), and autofluorescence of flavoproteins observed in the absence of substrate (*B*) in isolated and permeabilized cardiomyocytes, and by TMRE in permeabilized (skinned) cardiac fibres (*C*) and in ghost cardiac fibres (*D*). Inset in *D* shows electron microscopy of ghost fibres where removal of sarcomeric structures is clearly seen.

striation in the intact muscle (Fig. 2*D*). This is due to the localization of mitochondria almost exclusively at the level of the A-band of sarcomeres in intact cardiac muscle (Boudina *et al.* 2002). These ghost fibres, having diameters equal to those of cardiomyocytes, contain mitochondria and sarcoplasmic reticulum in close contact and they are perfectly organized and arranged by the cytoskeleton into regular structures (Fig. 2*D*). Nevertheless, the value of apparent $K_m$ for exogenous ADP is around 300 $\mu$M (Fig. 4), as in intact permeabilized cardiomyocytes with normal sarcomeres (Fig. 2*A* and *B*). Removal of myosin decreases the $Mg^{2+}$-ATPase activity about 5 times, but does not change the value of apparent $K_m$ for exogenous ADP (Fig. 4). Increasing the free $Ca^{2+}$ concentration up to 3 $\mu$M increases the $Mg^{2+}$-ATPase activity of ghost fibres by factor of 3 by activating the sarcoplasmic reticulum $Ca^{2+}$,$Mg^{2+}$-ATPase, but again no significant change in the values of apparent $K_m$ for exogenous ADP in the regulation of respiration *in situ* were found (Fig. 4). Thus, the apparent $K_m$ for exogenous ADP is independent of the intrinsic $Mg^{2+}$-ATPase activities of the preparation (Saks *et al.* 2001). This observation strongly favours the structural explanation of the phenomenon under discussion, and excludes the influence of ADP concentration gradients, which should be changed by $Mg^{2+}$-ATPase activities (see below).

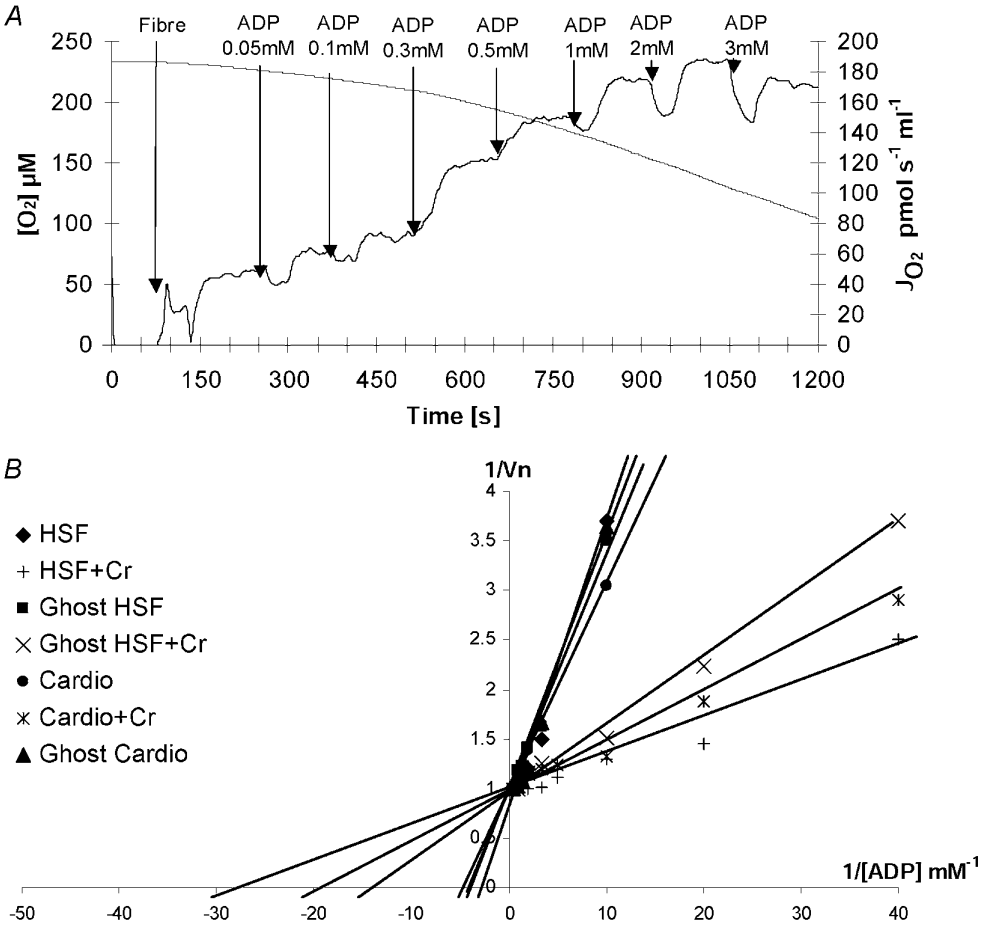


**Figure 3**

Kinetics of regulation of respiration by ADP in permeabilized cardiac cells and fibres *in situ*. *A* shows two recordings: the change of oxygen concentration with time (left ordinate axis) and its first derivation (right ordinate axis) that shows directly the rate of oxygen consumption by permeabilized cardiac fibres. *B* shows in double-reciprocal plots the kinetics of the regulation of mitochondrial respiration *in situ* in different cardiac cell preparations (HSF, heart skinned fibres; Cardio, isolated cardiac myocytes; and ghosts of both) without and with added creatine (Cr). Apparent $K_m$ for exogenous ADP is in the range 250–350 $\mu$M before addition of creatine. Creatine, due to coupled mitochondrial creatine kinase, decreases the apparent $K_m$ for ADP by strongly increasing the turnover of the adenine nucleotides in mitochondria. $V_n$ is the normalized rate of respiration, calculated as $\{v\,(\mathrm{ADP}) - v_0\}/V_{max}$, where $v$ (ADP) is the respiration rate at the given ADP concentration, $v_0$ is the respiration rate before ADP addition (state 2), and $V_{max}$ is the maximal respiration rate in a given experiment.

### Intramitochondrial volume changes as a possible factor in the modification of apparent $K_m$ for exogenous ADP

The technique of permeabilized cardiac cells and fibres has been very useful in two aspects: (1) for the elucidation of the mechanism of regulation of respiration in oxidative muscle cells *in vivo* (Kay *et al.* 2000; Garlid, 2001; Walsh *et al.* 2001), and (2) in human and clinical studies, where a very limited amount of biopsy material is available and the total population of mitochondria in the tissue sample has to be analysed (Kay *et al.* 1997*a*,*c*; Walsh *et al.* 2001). Furthermore, the apparent $K_m$ for exogenous ADP in the regulation of mitochondrial respiration is an important quantitative parameter of the intactness of the cell (Kay *et al.* 1997*a*,*c*; Rossi *et al.* 1998; Boudina *et al.* 2002). For the interpretation of all these data and for the correct use of the technique, it is important to know the cellular mechanism of the decreased affinity of mitochondrial respiration for ADP in cardiac cells *in situ*. The original explanation for the phenomenon given by Kummel (1988) and by our groups (Saks *et al.* 1991, 1993, 1994, 1995, 2001; Seppet *et al.* 2001) is based on the cellular organization, proximity of intracellular ATP producing and consuming systems and metabolic channelling. The value of the apparent $K_m$ for exogenous ADP decreases rapidly in ischaemic heart, in parallel with the opening of the mitochondrial outer membrane for cytochrome *c* release (Rossi *et al.* 1998). This parameter is well preserved (again in parallel with the intactness of the outer mitochondrial membrane) under conditions of cardioprotection by cardioplegia (Kay *et al.* 1997*a*,*c*), ischaemic preconditioning (Laclau *et al.* 2001) and by diazoxide, an opener of the ATP-dependent $K^+$ channel ($K_{ATP}$ channel), in particular in mitochondria (Dos Santos *et al.* 2002). The open question is whether the preservation of the mitochondrial outer membrane and intermembrane space structure is primary or secondary to mechanisms of cell protection. Dos Santos *et al.* have proposed, on the basis of the experiments with diazoxide, that the mitochondrial matrix volume, which is controlled by $K_{ATP}$ channels, may in its turn control the intermembrane space structure and permeability of the outer mitochondrial membrane, and thus the value of apparent $K_m$ for exogenous ADP (Dos Santos *et al.* 2002). In this case, low $K_m$ (ADP) for mitochondria *in vitro* is a result of the loss of $K^+$ during isolation, and mitochondria in permeabilized fibres are saved by the presence of the $K^+$ in solution B (Dos Santos *et al.* 2002). Indeed, they showed in experiments with isolated heart mitochondria *in vitro* that the increase of matrix volume in slightly hypertonic solution increased the apparent $K_m$. In close agreement with our earlier data they showed that decreasing osmolarity further disrupted the outer mitochondrial membrane and sharply decreased the apparent $K_m$ for exogenous ADP, as it was observed in permeabilized fibres from ischaemic-reperfused hearts. This clearly means that the apparent $K_m$ for exogenous ADP is related to controlled permeability of the outer mitochondrial membrane for adenine nucleotides. While this correctly emphasizes the connection between the value of the apparent $K_m$ for exogenous ADP and intactness of the mitochondrial outer membrane (see below), the changes in the intramitochondrial volumes are not yet sufficient to explain the phenomenon of high apparent $K_m$ for exogenous ADP in permeabilized heart cells. In isosmotic solution the apparent $K_m$ for ADP is always low in mitochondria *in vitro* in the presence of $K^+$ and high in permeabilized cells (Table 1). It is shown in this work that what is still absent in the theory of Dos Santos *et al.* is the importance of the interaction of mitochondria with other cellular structures, and thus the role of cell structural organization.

### A possible way of misinterpreting the experimental data on the apparent $K_m$ for exogenous ADP in cardiac cells *in situ* (to be avoided)

Another recent attempt to explain the high apparent $K_m$ values for exogenous ADP in mitochondria in cardiac muscle cells *in situ* was made by van Beek's group in Amsterdam (Kongas *et al.* 2002). This was an unsuccessful attempt to save the theory of cell metabolism as an homogeneous system of chemical reactions. The authors supposed that inside the muscle bundles all mitochondrial

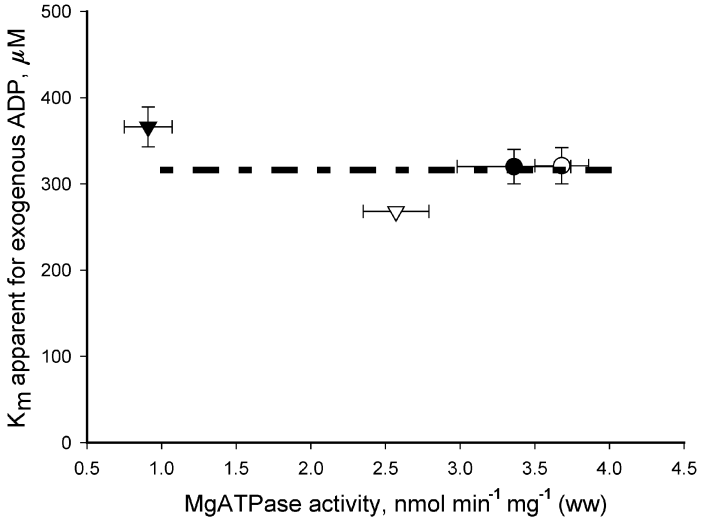


**Figure 4**

Apparent $K_m$ for ADP and ATPase activity for different fibres. For different $Ca^{2+}$ concentrations the $K_m$ for ADP is stable; on the other hand, absence of myosin ('ghost') decreases ATPase activity. The high apparent $K_m$ values for exogenous ADP are independent of the $Mg^{2+}$-ATPase activities. Skinned fibres: ●, 0 μM $Ca^{2+}$; ○, 0.4 μM $Ca^{2+}$. Ghost fibres: ▼, 0.4 μM $Ca^{2+}$; ▽, 3.0 μM $Ca^{2+}$.

enzymes and $Mg^{2+}$-ATPases are uniformly distributed over the bundle volume, and diffusion of ADP and ATP is rapid and corresponds to their diffusion in the homogeneous water bulk phase. Thus, the fibre bundle is modelled as one big 'well mixed bag' without any cells and without any structural organization of the intracellular medium. By computing the distribution of ADP, the authors found that significant ADP concentration gradients may exist between the medium and core of the bundle, but only for rather long diffusion distances, 40–50 $\mu$m, corresponding to bundles of about 100 $\mu$m in diameter. This minor result of calculations with their artificial model was taken, however, as evidence that the high values of apparent $K_m$ for exogenous ADP 'stem from' large concentration gradients between the large homogenous bundle core and the medium, which were found to depend reciprocally on the activity of homogeneously distributed $Mg^{2+}$-ATPase. The authors ignored the principal differences between the single, isolated cardiomyocytes and their artificial hypothetical 'homogeneous' bundle. In reality, values of the apparent $K_m$ for exogenous ADP higher than 200–300 $\mu$M are intrinsic characteristics of isolated cardiac cells (Kummel, 1988; Saks *et al.* 1991) or even their ghosts (Kay *et al.* 1997*a,b,c*) with diameters not exceeding 10–20 $\mu$m (see Figs 1, 2 and 3), and for these small diffusion distances the Brownian movement of ADP in the water phase is too rapid to allow any concentration gradient to be formed (Saks *et al.* 2001; Vendelin 2002). If applied for the diffusion distance of 8–10 $\mu$m from the medium into the core of the cell, the calculations of van Beek's group led to the same conclusion. Thus, the authors failed to correctly interpret even their own results. On the other hand, it is

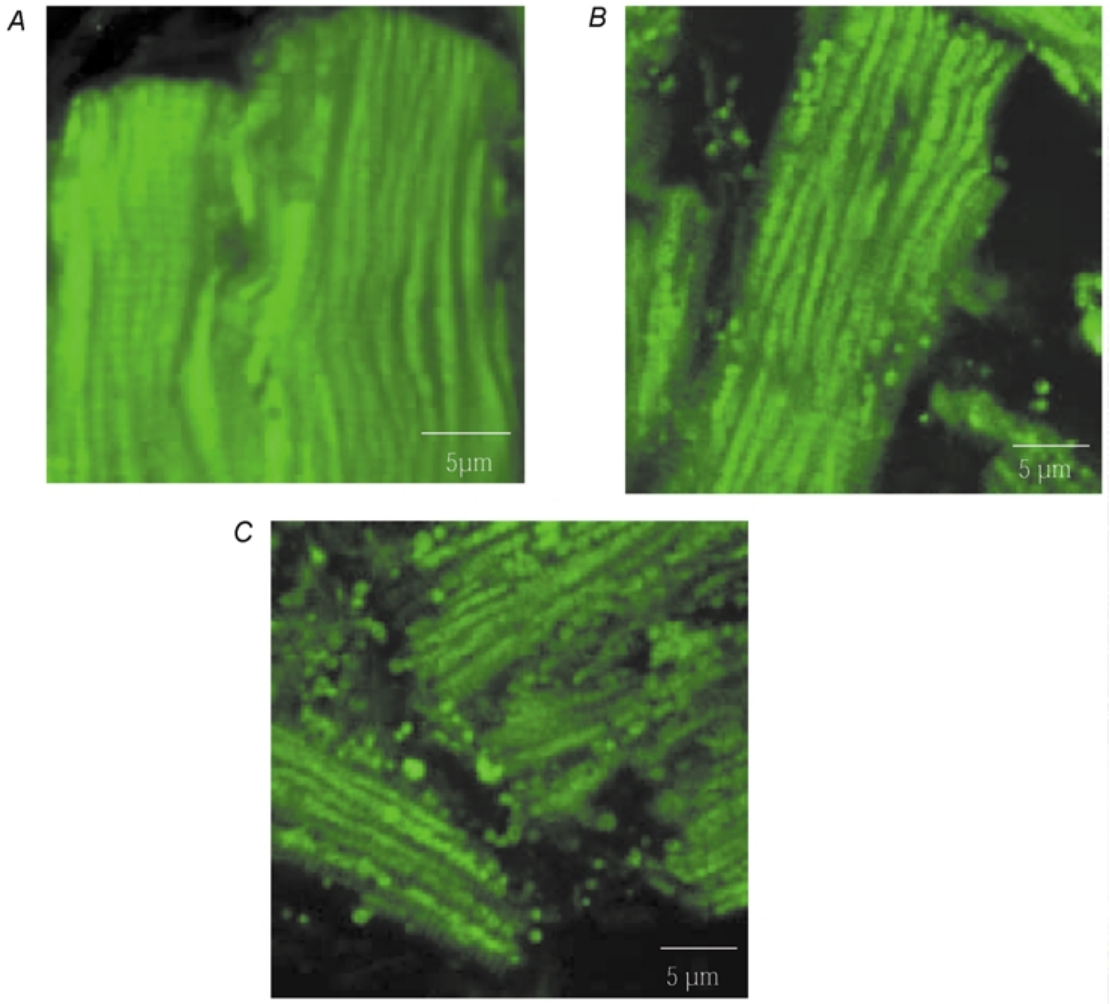


**Figure 5**

Confocal imaging of mitochondria in permeabilized cardiac fibres using a fluorescent probe MitoTracker Green FM. *A*, control permeabilized fibre: mitochondria are punctuated and very regularly arranged between myofibrils. *B*, after treatment for 5 min at 4 °C with 0.1 $\mu$M trypsin: the protease begins to disorganize the intracellular structure. *C*, after treatment with 5 $\mu$M trypsin: complete disorganization of intracellular structure is clear.

also well known that in fast twitch glycolytic muscle fibres, with the diameter around 80 $\mu$m, the apparent $K_m$ for exogenous ADP is low, 10–20 $\mu$M (Kuznetsov *et al.* 1996; Burelle & Hochachka, 2002). In solution B at pCa = 7 the overall $Mg^{2+}$-ATPase activities of the permeabilized cardiac and fast skeletal muscle fibres are close to each other, since the actomyosin system is not activated by calcium (this occurs at pCa = ~6), and thus, the phenomenon of a high apparent $K_m$ for exogenous ADP is tissue specific, of an intracellular nature and indeed does not depend on the diffusion distance (Kuznetsov *et al.* 1996; Kay *et al.* 1997*b*; Burelle & Hochachka, 2002). High apparent $K_m$ for exogenous ADP is also observed in ghost fibres after extraction of myosin (Saks *et al.* 1993; Kay *et al.* 1997*b*) and decreasing the intrinsic $Mg^{2+}$-ATPase activity several times (Fig. 4). This basic observation further supports the conclusions that there are no significant ADP concentration gradients between the medium and core of the cells (these gradients should be changed by $Mg^{2+}$-ATPase activities) and that apparent $K_m$ for exogenous ADP is independent of diffusion distance. Another serious artefact that Kongas *et al.* (2002) produced was to add excessive hexokinase activity to increase the cellular $Mg^{2+}$-ATPase activity. Adding glucose and a very high amount of hexokinase (about 100 i.u. $ml^{-1}$, in contrast to 4 i.u. $ml^{-1}$ used in our experiments) as a model ATPase, they found a decrease in the apparent $K_m$ for ADP in permeabilized fibres. Again, the authors ignored the protein–protein interactions in these highly concentrated protein solutions, the sticking of hexokinase to the cytoskeleton network and to the outer mitochondrial membrane, and thus the formation of local futile coupled reactions of increased ADP–ATP turnover. In some aspects, this artificial and futile coupled system is (functionally) analogous to the physiologically important coupled creatine kinase reaction (see above). This effect of very high concentrations of hexokinase on apparent $K_m$ (ADP) was observed earlier (E. Boehm, V. Veksler & R. Ventura-Clapier, personal communication). Figure 4 shows very clearly that the apparent $K_m$ for exogenous ADP is independent of the $Mg^{2+}$-ATPase activity, and thus, the decrease in apparent $K_m$ found by van Beek's group (Kongas *et al.* 2002) is most probably simply a result of local futile coupled cycle formation. Further, trying to explain the equal values of apparent $K_m$ for exogenous ADP in permeabilized and in ghost fibres with very different $Mg^{2+}$-ATPase activities, it was suggested that after removal of myosin the diffusion coefficient of ADP in the homogeneous water phase increases, in conflict with the first law of thermodynamics. If there is any positive value in this recently published 'theory of artefacts' (Kongas *et al.* 2002), it is the clear conclusion that the homogeneous system of enzymes and substrates as a well-mixed bag is not a suitable model to explain the energy metabolism in the living cell, not even in the simplest ghost fibre cells, and thus is not applicable.

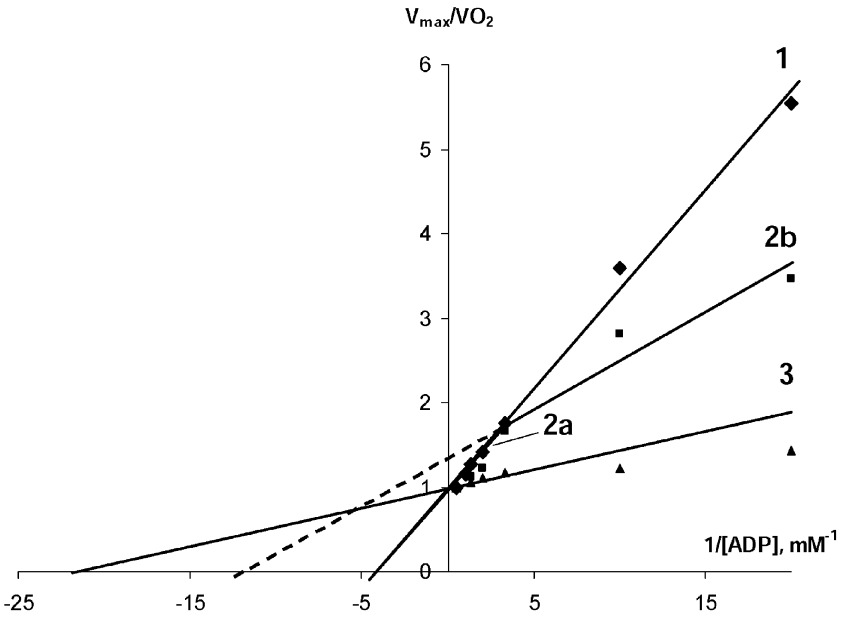


**Figure 6**

Effect of trypsin on apparent $K_m$ for ADP of mitochondrial respiration in permeabilized cardiac fibres (presentation of data in Lineweaver-Burk plots). 1 (◆), control: apparent $K_m$ for exogenous ADP is 250–300 $\mu$M. 2 (■), fibres treated with 100 nM trypsin (5 min at 4 °C): there are at least two populations (a,b) of mitochondria with different apparent $K_m$ values for exogenous ADP. 3 (▲), fibres treated with 5 $\mu$M trypsin: apparent $K_m$ is between 40 and 50 $\mu$M; kinetically homogeneous population. $V_{max}/V_{O_2}$ represents $1/V_n$, see Fig. 3.

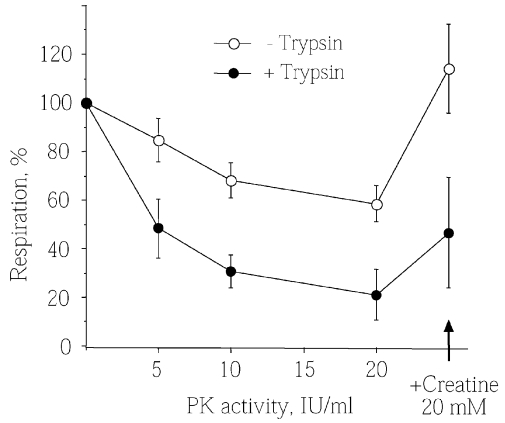


**Figure 7**

Changes of metabolic channelling of endogenous ADP from $Ca^{2+}$,$Mg^{2+}$-ATPases to mitochondria visualized by the PK–PEP competitive enzyme method, before and after treatment of permeabilized cardiac fibres with trypsin, leading to the disorganization of the regular arrangement of mitochondria. ATP was added to the final concentration of 2 mM to activate endogenous ADP production inside the cells. The respiration rates are shown as a percentage of maximal respiration rates (ATP, 2 mM) before addition of phosphoenol pyruvate, PEP (5 mM) and increasing amounts of pyruvate kinase (PK). ○, control permeabilized fibres. ●, permeabilized fibres treated with 5 mM trypsin for 15 min at 4 °C. At the end of the experiment, creatine was added to a concentration of 20 mM. Note that treatment with trypsin increases the accessibility of endogenously produced ADP to the PEP–PK system.

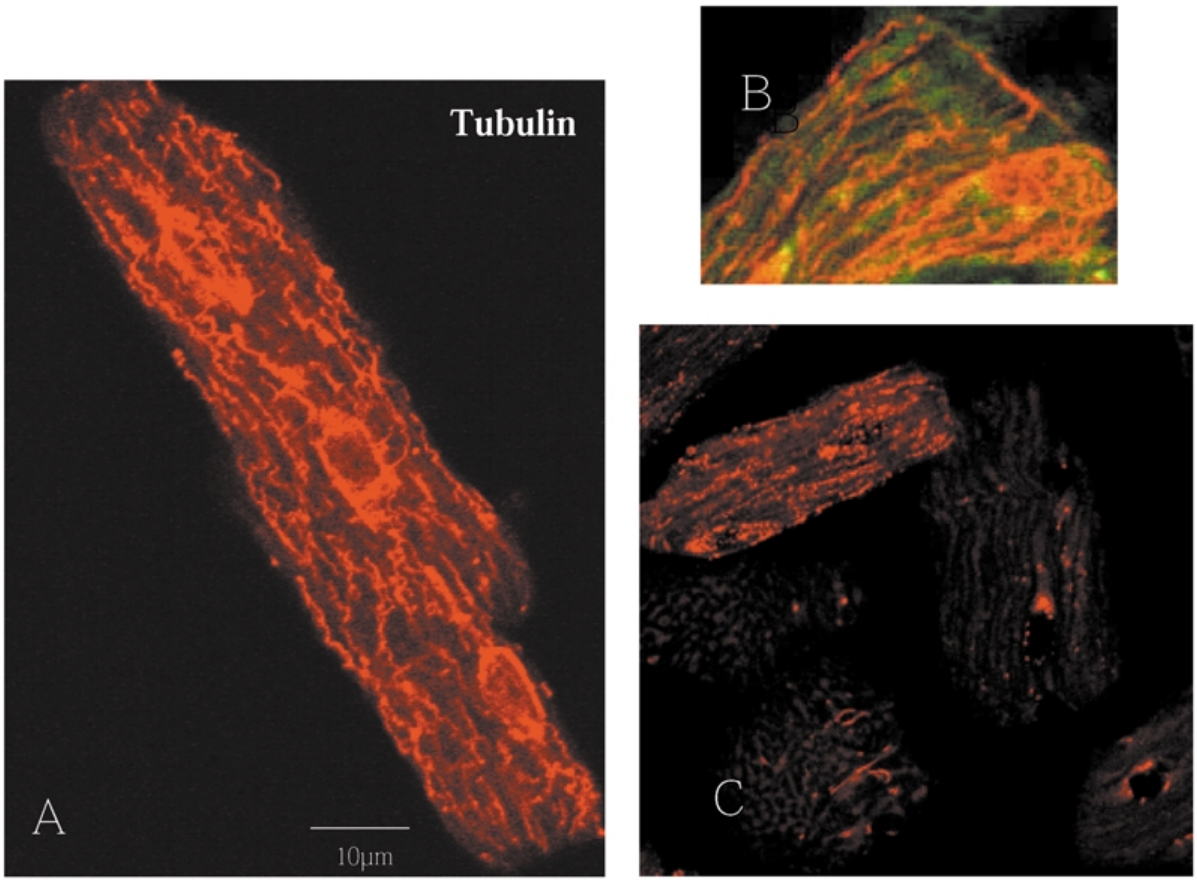


**Figure 8**

Confocal imaging immunofluorescence of microtubular network in cardiomyocytes. *A*, microtubule network in control cardiomyocyte. *B*, double labelling immunofluorescence of mitochondria and tubulin in control cardiomyocyte. The green colour is that of MitoTracker Green FM associated with mitochondrial membranes, and red colour is the staining for tubulin. *C*, effect of trypsin treatment (5 min, 1 µM at 4 °C) on the intracellular organization of microtubular network of cardiomyocyte: tubulin labelling disappears.

This conclusion is not, however, unexpected and at the present time even becoming universal (Saks *et al.* 1998*a*; Weiss & Korge, 2001; Kaasik *et al.* 2001; Greenhaff, 2001).

**The cytoskeletal network is important for mitochondrial arrangement and regulation in muscle cells *in situ***

Thus, we have the following firm experimental data for the interpretation of the differences in the kinetics of the regulation of mitochondrial respiration in cardiac cells *in vitro* and *in vivo*.

(1) This phenomenon is of an intracellular nature, is tissue specific and is observed in cardiac cells, in hepatocytes, in oxidative slow twitch skeletal muscle cells, but not in fast-twitch skeletal muscle cells.

(2) Hyposmotic shock, by disrupting outer mitochondrial membrane, decreases the apparent $K_m$ for exogenous ADP. The same effect is seen in acute or chronic ischaemia where it could be prevented by cardioprotective measures. This shows that the integrity of the mitochondrial outer membrane is involved in the phenomenon.

(3) The kinetics of the regulation of mitochondrial respiration *in situ* depends upon the source of ADP and is very different for exogenous and endogenous ADP (Seppet *et al.* 2001).

(4) Finally, the proteolytic treatment of the permeabilized cells described below shows clearly the participation of cytoplasmic proteins in the regulation of mitochondria *in situ*.

Figure 5*A* shows confocal imaging of mitochondria in permeabilized cardiac fibres using a fluorescent probe MitoTracker Green FM. Once again, a very regular arrangement of mitochondria can be seen between myofibrils in the skinned cardiac fibres, in good agreement with many earlier observations (Duchen, 1999; Saks *et al.* 2001). This regular arrangement is rapidly disorganized by the treatment of fibres for 5 min at 4 °C with 1–5 $\mu$M trypsin (Fig. 5*B* and *C*). Figure 6 shows that, in parallel, the apparent $K_m$ for ADP progressively decreased to very low values of around 40–50 $\mu$M. At a very low trypsin concentration (100 nM), two or more different populations of mitochondria, and at higher trypsin concentrations, only one homogeneous population of mitochondria with very low apparent $K_m$ for exogenous ADP was seen (Fig. 6). Figure 7 shows that the proteolytic treatment and disorganization of the intracellular arrangement of

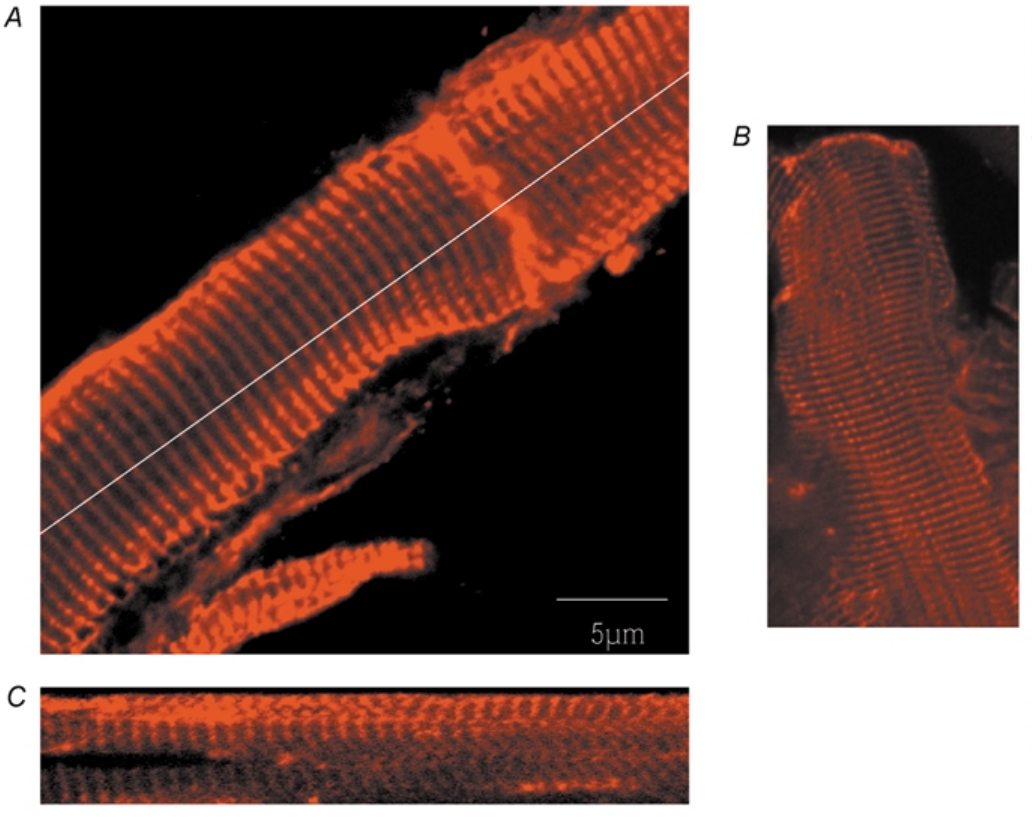


**Figure 9**

Confocal immunofluorescence imaging of desmin in cardiomyocytes. *A*, control cardiomyocyte: desmin was mostly associated with the Z-lines. *B*, desmin was not affected by trypsin treatment. Indeed, the same structure of desmin is still present in skinned fibres after 1 $\mu$M trypsin. *C*, z-scan of the cardiomyocyte in *A* to see if desmin staining is the same throughout the cardiomyocyte.

mitochondria significantly increases the accessibility of the endogenous ADP to the added trapping system – pyruvate kinase and phosphoenol pyruvate (Gellerich & Saks, 1982). Indeed, in this experiment, respiration was stimulated by endogenous ADP produced from added exogenous ATP. While, this stimulation was only inhibited by ~30 % in the presence of the ADP-trapping system, the system was more efficient at trapping ADP and decreasing respiration after the proteolytic treatment of the permeabilized fibres (5 $\mu$M trypsin, 5 min at 4 °C). Taken together, all these results show that the regular intracellular arrangement of mitochondria, high apparent $K_m$ for exogenous ADP and channelling of endogenous ADP to mitochondria are most probably related phenomena, due to the presence of proteins sensitive to trypsin. These proteins seem to be connected to the cytoskeleton, since similar high $K_m$ values for exogenous ADP are also observed for 'ghost' cells and fibres, from which myosin has been extracted and which contain mostly mitochondria, sarcoplasmic reticulum and cytoskeletal structures (see above). Therefore, the next intriguing questions are: (1) What happens to the cytoskeleton during this short, selective treatment of fibres with trypsin, which disorganizes the mitochondrial arrangement in the cells and strongly decreases the apparent $K_m$ for exogenous ADP? (2) Which of the many cytoskeletal components might be responsible for mitochondrial arrangement and control of function?

In this work, we considered three important components of cytoskeleton: the microtubular network, desmin and plectin.

The microtubular network. Microtubules are essentially composed of tubulin (a and b heterodimers), and their assembly and function are regulated by microtubule-associated proteins, the major classes of which are the kinesin, dynein and the structurally associated proteins (tau, MAPs), which modulate tubule stability and spatial arrangement. The microtubules exist in an equilibrium between polymerized and depolymerized states (Rappaport *et al.* 1998). Cytoskeletal networks of microtubules that

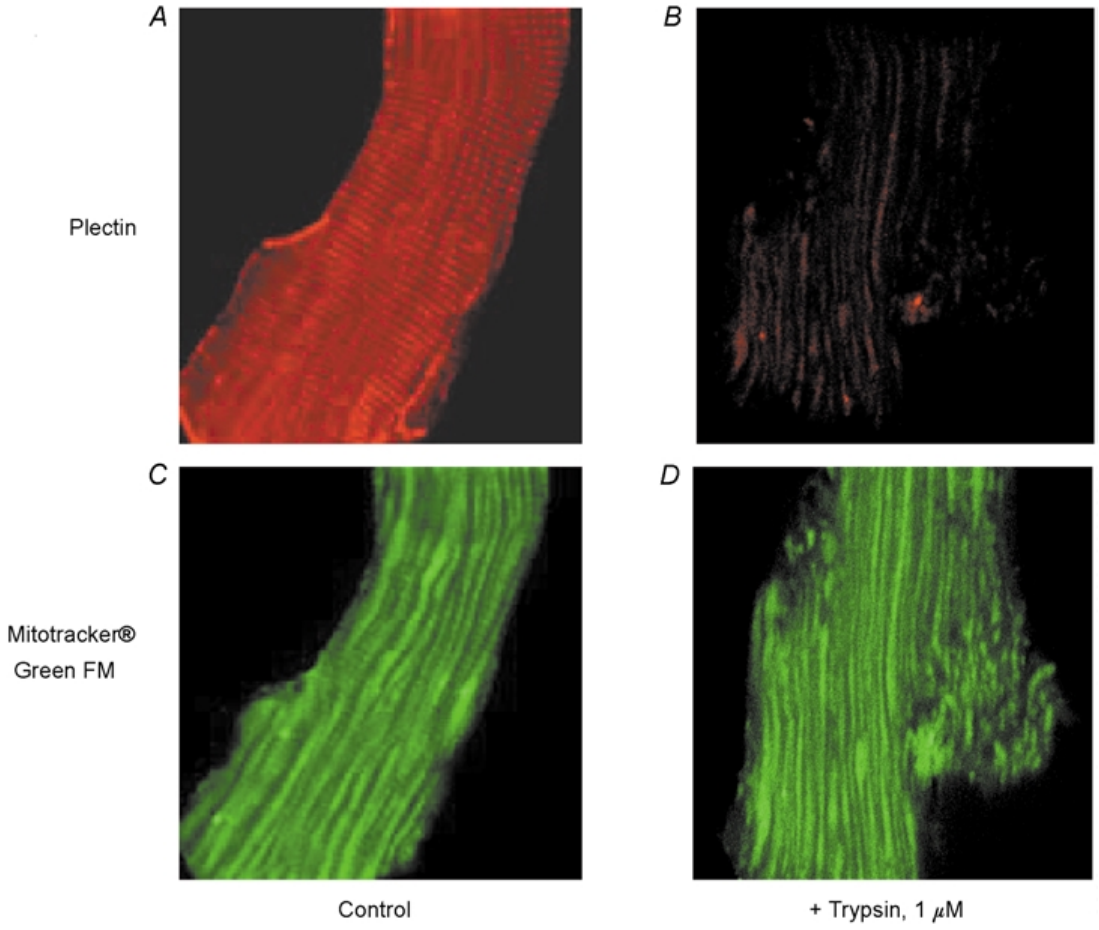


**Figure 10**

Confocal imaging immunofluorescence of plectin in cardiomyocytes. In control cardiomyocytes, labelling of plectin (*A*) gives the same staining as desmin, showing that plectin co-localized with desmin. In *C* there is still the same staining for MitoTracker Green FM as in Fig. 5*A*. After trypsin (1 $\mu$M) treatment (*B* and *D*), the regular mitochondrial arrangement is changed and plectin has totally disappeared.

maintain the cellular morphology also provide the rails for organelle trafficking (Leterrier *et al.* 1994; Yaffe, 1999). By permeabilizing the sarcolemma, two factors could destabilize microtubular network. First, dilution of the medium could provoke depolymerization of microtubules. Second, through actin, microtubules are linked to cellular membrane; thus, the permeabilization of the cell membrane could collapse this network. However, Fig. 8*A* shows that the microtubular network is extremly dense and intact in the cardiomyocytes, even if the cells were permeabilized and kept in solution B at 4 °C before staining. Figure 8*B* shows double labelling of the microtubular network in cardiomyocytes and mitochondria: it seems that the mitochondria are wrapped into the network. Thus, in our conditions, the microtubular network was maintained intact in permeabilized cells, probably because of some structural proteins associated with tubulin, and possibly because of the ionic composition of the medium. However, Fig. 8*C* shows that after selective treatment by trypsin, which disorganizes the regular arrangement of mitochondria (Fig. 5), the microtubular network progressively disappears with increasing concentration of protease. A parallel decrease of Km for ADP after trypsin treatment and the disappearance of immunolabelling of the microtubular network may suggest the possible role of microtubules in the regulation of mitochondrial respiration. Indeed, mild treatment of mitochondria by trypsin has been shown to decrease the tubulin binding, suggesting that protein component(s) of membranes are involved in the interaction of tubulin with mitochondria (Bernier-Valentin & Rousset, 1982; Saetersdal *et al.* 1990).

**Desmin.** The second protein studied is the main cytoskeletal protein in cardiac and skeletal muscle. Indeed, the muscle-specific intermediate filament (IF) protein desmin is expressed in all striated and smooth muscle tissues. Ultrastructural studies have also suggested the potential association of IFs with mitochondria. According to Milner *et al.* (2000), desmin plays a significant role in mitochondrial positioning in cardiac and skeletal muscle. According to our previous results (Kay *et al.* 1997*b*), this protein may not interact directly with mitochondria to regulate the mitochondrial function *in vivo*, but nevertheless mitochondrial clustering in desmin-deficient mice decreased the apparent $K_m$ for exogenous ADP (Kay *et al.* 1997*b*; Milner *et al.* 2000), showing the importance of mitochondrial intracellular arrangement for regulation of their function.

Figure 9 shows the classical staining of desmin in cardiac fibres (*A*), and that desmin was not significantly affected by trypsin treatment (*B*). This shows, therefore, that desmin may not be the direct candidate for the control of mitochondrial function. This is in agreement with our observations on desmin-deficient mice, where we still observed a population of mitochondria with a high apparent $K_m$ for ADP, and another with a very low apparent $K_m$, obviously because of mitochondrial clustering as a result of a weakening of cell structure in the absence of desmin (Kay *et al.* 1997*b*).

**Plectin.** Plectin is a cytoskeletal protein of more than 500 kDa that forms a central parallel α-helical coiled rod domain flanked by globular domains, thus providing a

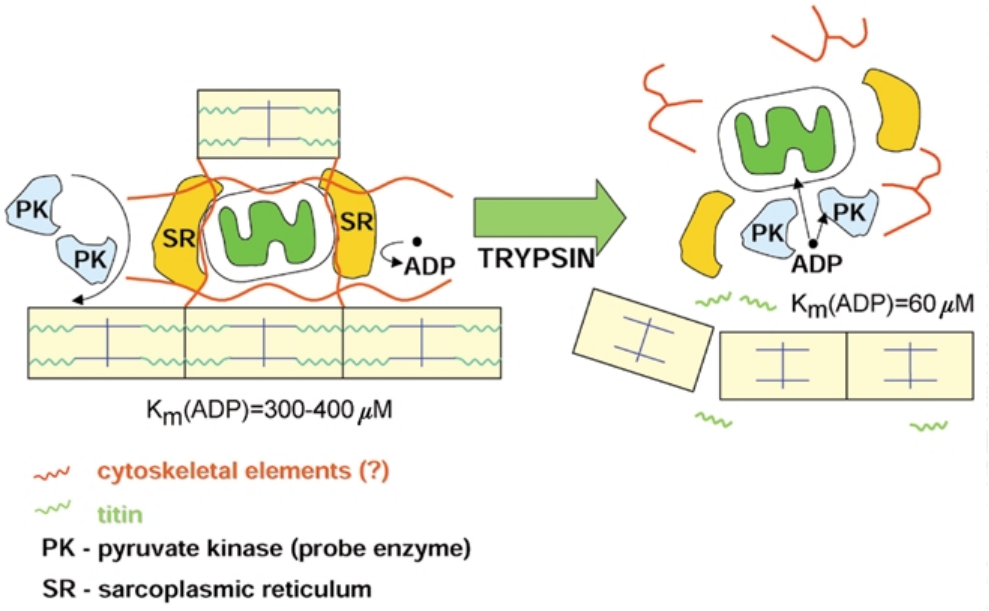


**Figure 11**

Role of cytoskeleton in the organization of mitochondria into functional complexes with sarcoplasmic reticulum (SR) and sarcomeres, i.e. into intracellular energetic units, ICEUs (Saks *et al.* 2001). PK, pyruvate kinase. Proteolytic treatment with trypsin results in the collapse of the cytoskeleton and disorganization of the regular arrangement of mitochondria within the cells. For further explanation see text.

molecular backbone ideally suited to mediate the protein's interactions with an array of other cytoskeletal elements (Steinbock & Wiche, 1999). Interestingly, plectin was often found to localize at the periphery of Z-discs during the actual alignment of neighbouring myofibrils, and an obvious cross-striated plectin-stained pattern was observed before desmin was localized in the Z-disc region (Schroder *et al.* 2000). The association of plectin with Z-discs is an early event in the lateral alignment of myofibrils that precedes the formation of the intermyofibrillar desmin cytoskeleton (Schroder *et al.* 2000). Plectin may have a central role in the structural and functional organization of the intermediate filament cytoskeleton in skeletal muscle. Through plectin threads, desmin intermediate filaments form lateral linkages among adjacent Z-discs, preventing individual myofibrils from disruptive contraction and ensuring effective force generation (Hijikata *et al.* 1999). There is a direct interaction of plectin with desmin via its C-terminal IF-binding domain. As a cytolinker protein associated with mitochondria and desmin IFs, plectin could play an important role in the positioning of mitochondrial organelles in striated muscle tissues (Reipert *et al.* 1999). According to our results, plectin co-localizes with desmin but trypsin treatment has not the same effect on both proteins. Indeed, desmin seems not to be affected by the protease whereas plectin was cleaved and its staining disappeared after treatment with 1 $\mu$M trypsin (Fig. 10).

Plectin links desmin with mitochondria, but plectin interacts with a variety of proteins including MAP-2 (microtubule associated protein) and actin. Microtubules interact with mitochondria and of course are associated with MAPs. Therefore, it is not yet clear if only the microtubular network and plectin, or mostly other cytolinker proteins are important for the arrangement and control of mitochondria.

In general, all data presented and analysed in this work lead us to conclude that there is a new and important intracellular factor involved in the metabolic regulation of mitochondrial respiration and energy fluxes. This is the structural organization of the cell, evidently by the cytoskeleton. This organization is a basis for effective cross-talk between different subcellular systems of energy conversion. The conclusions of our studies are summarized in Fig. 11: some components of the cardiac cell cytoskeleton fix mitochondria, sarcoplasmic reticulum and one sarcomere into functional complexes where endogenous ADP is channelled by organized metabolic networks to mitochondria (Dzeja *et al.* 1998, 1999; Saks *et al.* 1998*a*, 2001) in order to precisely regulate the free energy conversion rates with respect to workload, thus explaining the important phenomenon of the metabolic stability of the heart (Saks *et al.* 1995). This explains why endogenous ADP is preferentially channelled to mitochondria and why it becomes more accessible for the PK–PEP system after selective proteolysis which results in the collapse of the cytoskeleton (Fig. 7). This also explains the low affinity of the system for exogenous ADP, the diffusion of which to mitochondria may be locally restricted because of the structural organization of these complexes and probably by the limitation of the permeability of the mitochondrial outer membrane for this substrate. Both limitations seem to be eliminated by the collapse of the cytoskeleton by proteolysis.

Clearly, the identification of the precise nature of the links between mitochondria and cytoskeleton still need further intensive studies. These are the exciting tasks for a new research direction – structural bioenergetics.

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