Intracellular energetic units in red muscle cells

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The kinetics of regulation of mitochondrial respiration by endogenous and exogenous ADP in muscle cells *in situ* was studied in skinned cardiac and skeletal muscle fibres. Endogenous ADP production was initiated by addition of MgATP; under these conditions the respiration rate and ADP concentration in the medium were dependent on the calcium concentration, and 70–80 % of maximal rate of respiration was achieved at ADP concentration below 20 μM in the medium. In contrast, when exogenous ADP was added, maximal respiration rate was observed only at millimolar concentrations. An exogenous ADP-consuming system consisting of pyruvate kinase (PK; 20–40 units/ml) and phosphoenolpyruvate (PEP; 5 mM), totally suppressed respiration activated by exogenous ADP, but the respiration maintained by endogenous ADP was not suppressed by more than 20–40 %. Creatine (20 mM) further activated respiration in the presence of ATP and PK + PEP. Short treatment with trypsin (50–500 nM for 5 min) decreased the apparent K_m for exogenous ADP from 300–350 μM to 50–60 μM, increased inhibition of respiration by PK + PEP system up to 70–80 %, with no changes in MgATPase activity and maximal respiration rates. Electron-microscopic observations showed detachment of mitochondria and disordering of the regular structure of the sarcomere after trypsin treatment. Two-dimensional electrophoresis revealed a group of at least seven low-molecular-mass proteins in cardiac skinned fibres which were very sensitive to trypsin and not present in glycolytic fibres, which have low apparent K_m for exogenous ADP. It is concluded that, in oxidative muscle cells, mitochondria are incorporated into functional complexes ('intracellular energetic units') with adjacent ADP-producing systems in myofibrils and in sarcoplasmic reticulum, probably due to specific interaction with cytoskeletal elements responsible for mitochondrial distribution in the cell. It is suggested that these complexes represent the basic pattern of organization of muscle-cell energy metabolism.

Key words: ADP, ATPases, compartmentation, mitochondria, myocytes.

INTRODUCTION

The fundamental mechanisms of mitochondrial respiration, its regulation and ATP synthesis have been described in *in vitro* studies of isolated mitochondria or their components [1,2]. However, the nature of a feedback signal from ATPases in myofibrils and in cellular and subcellular membranes to mitochondria *in vivo*, responsible for precise matching of the free-energy production to its demand is still not known. In the heart the workload and respiration rate may be increased by more than an order of magnitude at practically constant levels of phosphocreatine, creatine and ATP [3,4]. This has led to the conclusion that there is no metabolic feedback regulation of mitochondrial activity in the heart, and to an alternative explanation of regulation of respiration by Ca^{2+}, in parallel with regulation of contraction (so-called 'parallel activation of respiration') [5,6]. However, there is still a linear relationship between workload and oxygen uptake after inhibition of calcium uptake into mitochondria [7,8]. Application of fluorescence spectroscopy to study the reduction–oxidation state of mitochondrial NAD^+/NADH in intact heart muscle at different pacing rates showed, in fact, that the regulatory signal for mitochondrial respiration is downstream of the respiratory chain [9]. And, in any case, it is the ADP produced in myofibrils during contraction, or related compounds such as creatine that should be rephosphorylated at any workload and serve as phosphate acceptor. Their roles in the regulation of respiration need to be elucidated quantitatively. An equally important problem, which is at present far from being solved, is the mechanism of acute heart insufficiency in ischaemia or anoxia, when the contractile force declines rapidly in the presence of high amounts of ATP in the cells [10,11]. Because of the obscurity of its solution, the scientific community even seems to have lost interest in this important problem.

Interpretation of *in vivo* data is complicated because of subcellular compartmentation of enzymes and metabolites and specific organization of the cell interior. We have used the permeabilized (skinned) cell technique, which allows us direct access to different subcellular compartments in the cell *in situ*, to investigate the intracellular mechanisms of regulation of respiration [12–16]. These studies have shown that mitochondrial respiration may be controlled by a local production of ADP by mitochondrial creatine kinase in the intermembrane space of mitochondria. In the present study we have developed this direction of research further to investigate the relative importance of different intracellular sources of ADP for regulation of respiration. The results show close structural and functional interactions between mitochondria, the sarcoplasmic reticulum and sacromeric structures. It is concluded that, in the muscle cells *in vivo*, these subcellular structures are organized, probably by proteins associated with cytoskeleton, into discrete functional complexes which may be called 'intracellular energetic units'

Abbreviations used: PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase; ICEU, intracellular energetic unit; DTT, dithiothreitol; VDAC, voltage-dependent anion channel.

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(ICEUs) and whose activities are synchronized in normal cells. Within these units metabolites are transfered both by channelling and facilitated diffusion, and their local concentrations may differ from those in the bulk-water phase of cytoplasm.

EXPERIMENTAL

Animals

Male Wistar rats weighing 300–350 g were used in 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).

Preparation of skinned muscle fibres

Skinned fibres were prepared from endocardium of left ventricles of rat hearts according to the method described in detail previously [14].

Ghost fibres

Ghost fibres (skinned fibres deprived of myosin) were produced by washing saponin-skinned fibres with solution C for 10 min, extraction of myosin with solution D for 30 min, and then washing twice in solution B for 5 min and kept in the third portion of solution B (for not more than 3–4 h). The compositions of solutions A–D are given in the subsection 'Solutions' below.

Isolation and culturing of adult cardiac myocytes

This was carried out as described by Kay et al. [15].

Isolation of heart mitochondria

This was carried out as described previously [16].

Imaging of mitochondria in skinned cardiac fibres

The cardiac fibres were gently stirred in solution A in the presence of the mitochondrion-selective dye Mito Tracker ® Red CMXRos (Molecular Probes, Inc., Eugene, OR, U.S.A.) (200 nM) in the dark for 30 min. Thereafter the fibres were washed three times in above mentioned medium without dye for 15 min by stirring, to minimize background fluorescence. The stained fibres were incubated in HistoPrep 10% prechilled buffered 10% formalin solution (Fisher Scientific, Pittsburgh, PA, U.S.A.), containing formaldehyde (4%), methyl alcohol (1%) and phosphate buffer for 15 min. Thereafter the fibres were placed on and attached to the specimen glass with a drop of glycerol/PBS (mixed 1:1), and covered by a coverslip. All procedures were performed at 20 °C. The mitochondria were revealed and scanned using a MRC 1024 Bio-Rad laser confocal microscope with the PlanApo 60 × /1.40 oil objective (Olympus, Tokyo, Japan). The specimen was illuminated by krypton/argon laser (15 mW) light (568 nm) and the emitted light signal was filtered by a 605DF32 filter and collected according to the Kalman method using the Bio-Rad aquisition system.

Electron microscopy

Muscle tissue probes were fixed in 0.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in the same buffer. After dehydration with ethanol and acetone, the specimens were embedded in Epon 812. Thin sections were stained with uranyl acetate followed by lead citrate and examined in Tecnai 10 Electron Microscope (Philips, FEI Company, Eindhoven, Netherlands) at 100 kV.

Two-dimensional electrophoresis

Sample preparation

Skinned fibre samples of 20 mg were washed in 50 mM Tris/HCl (pH 7.1)/100 mM KCl/6 mM EDTA/5.8 μM benzamidine/2.1 μM leupeptin for 20 s, pulverized after having been frozen with liquid nitrogen and solubilized in 100 μl of buffer which contained 9 M urea, 25 mM Tris/HCl (pH 7.1), 50 mM KCl, 3 mM EDTA, 2.9 mM benzamidine, 70 mM dithiothreitol (DTT) and 2% (v/v) ampholyte (Servalyte; pH 2–4; Serva, Heidelberg, Germany). Finally, 2 μl of additional protease inhibitors (5 μM pepstatin and 50 mM PMSF) were added. The resulting protein concentration was approx. 20 μg/μl.

Two-dimensional gel electrophoresis and data analysis

The two-dimensional electrophoresis of proteins with carrier ampholytes and the data analysis of gels have been described previously [17–20]. Protein (150 μg) was loaded on to each gel. The gel size was 23 cm × 29 cm × 0.15 cm. The silver-stained gels [18] were digitized at 176 μm resolution by the use of a white light scanner (Howtek Inc., Hudson, NH, U.S.A.), and analysed for qualitative and quantitative variations in their protein patterns by the use of the PDQUEST [19,20] software package (Protein and DNA Imageware Inc., New York, NY, U.S.A.).

Determination of the kinetics of respiration regulation by ADP in skinned fibres and cardiomyocytes

The rates of oxygen uptake were recorded by using the two-channel high-resolution respirometer (Oroboros Oxygraph; Paar KG, Graz, Austria) or Yellow Springs Instruments (Yellow Springs, OH, U.S.A.) Clark oxygen electrode in solution B, containing respiratory substrates (see below) and 2 mg/ml of BSA. Determinations were carried out at 25 °C and the solubility of oxygen was taken as 215 nmol/ml [16]. If not shown otherwise, the respiration rates were expressed in nmol of O_2/min per mg dry weight of fibres. The total volume was 2 ml.

In some experiments the respiration rates were measured in a Ca^{2+}-free solution of the following composition: 250 mM sucrose, 20 mM Hepes (pH 7.1), 0.5–1 mM EGTA, 4 mM $MgCl_2$, 3 mM K_2HPO_4, 5 mM glutamate and 2 mM malate and 2 mg/ml BSA (fatty-acid-free). In some experiments Mg^{2+} was also omitted from this solution.

Determination of the flux of ADP produced in ATPase reactions

The skinned fibres of rat heart were prepared as described above, except that the washing medium B contained 5 mg/ml BSA. The role of mitochondria in rephosphorylation of ADP produced in ATPase reactions were estimated as a decrease in the flux through the external system of ATP regeneration [phosphoenolpyruvate (PEP)–pyruvate kinase (PK)–pyruvate–lactate dehydrogenase (LDH) after switching to operation of the mitochondrial oxidative phosphorylation. Approx. 3 mg of skinned fibres were incubated in the spectrophotometric (Perkin–Elmer Lambda 900) cuvette containing medium B complemented with 5 mg/ml BSA, 5 mM PEP, 20 units/ml PK, 20 units/ml LDH and 0.24 mM NADH at 25 °C. The medium was continuously stirred by using the magnetic stirrer operated by the Variomag® Telemodul (H + P Labortechnic GmbH, Oberschlessheim, Germany). The changes in A_{340} were measured before and after addition of the 1 mM ATP, as well as after subsequent additions of the substrates (10 mM glutamate and 2 mM malate) and 98 μM atractyloside. The reaction rate was estimated from the stable and linearly time-dependent portions of recordings. Care was

taken that neither of the coupled enzyme-system components was limiting the rate of ATPase reaction. The total volume of the reaction mixture was 1.5 ml.

Determination of ADP concentration in the reaction medium

The oxygraphic samples were used immediately for the HPLC studies or frozen in liquid nitrogen and stored at −80 °C until ADP concentration determination for not longer than 2 weeks.

Perchloric acid extraction

Perchloric acid 60 % (100 μl) was added to oxygraphic samples (600 μl) in order to precipitate all proteins (BSA) present in the respiratory medium. Then the samples were centrifuged at 17000 ***g*** for 10 min at 4 °C. The supernatants (600 μl) were neutralized by a 2 M solution of $KHCO_3$ (420–470 μl depending on the samples). The samples were spun once more at 17000 ***g*** for 10 min (4 °C) and the supernatants loaded directly on to an HPLC system. These neutralized extracts can be stored during a few weeks at −20 °C without degradation of adenine nucleotides.

Chromatography

The separation and determination of adenine nucleotides in chromatographic samples was performed by HPLC making use of the spectrophotometric detector at 253 nm. The C_{18} reversed-phased column (Nucleosil C18 100A; 4.6 mm × 250 mm, 5 μm; from Phenomenex, Hösbach, Germany) along with the DuPont 8800 HPLC equipment was used. The column was equilibrated with 100 mM KH_2PO_4 buffer (pH 6.0) and, after injection of the sample, eluted with the same buffer solution containing 5 % methanol. Elution of the ATP, ADP and AMP standards gave the retention times 2.99, 3.56 and 6.43 min respectively at an elution rate of 2 ml/min. Calibrations were performed according to the peak areas of the standard mixtures of low variable amounts of ATP (0–10 μmol) at a constant amount of ADP (100 μmol) and vice versa.

Creatine concentration determination

The total creatine concentration was determined in supernatants after treatment of cardiomyocytes with saponin or Triton X-100 and centrifugation for 5 min at 1000 ***g***. The assay was performed by a colorimetric diacetyl α-naphthol method after hydrolysis of phosphocreatine in acidic medium and neutralization [21].

LDH determination

The activity of LDH in the supernatants after treatment of cardiomyocytes with saponin or Triton X-100 was assayed as described previously [22].

Solutions

Solution A contained, in mM: CaK_2EGTA, 2.77; K_2EGTA, 7.23; $MgCl_2$, 6.56; DTT, 0.5; potassium Mes, 50; imidazole, 20; taurine, 20; Na_2ATP, 5.3; phosphocreatine, 15; (pH 7.1, adjusted at 25 °C). Solution B was modified in comparison to that described in [14] and contained, in mM: CaK_2EGTA, 1.9; K_2EGTA, 8.1; $MgCl_2$, 4; DTT, 0.5; potassium Mes, 100; imidazole, 20; taurine, 20; K_2HPO_4, 3; pyruvate, 5 (or glutamate, 5) + malate, 2; (pH 7.1, adjusted at 25 °C). These modifications were made to complex all ATP and ADP into MgATP and MgADP under all experimental conditions. Solution C contained, in mM: potassium Hepes, 50; $MgCl_2$, 10; ATP, 10; DTT, 0.5; taurine, 20; potassium Mes, 80; (pH 7.1). Solution D contained, in mM: KCl, 800; potassium Hepes, 50; $MgCl_2$, 10; ATP, 10; DTT, 0.5; taurine, 20; (pH 7.1). Solution KCl contained, in mM: KCl, 125; Hepes, 20; glutamate, 4; malate, 2; magnesium acetate, 3; KH_2PO_4, 5; EGTA, 0.4; and DTT, 0.3; (pH 7.1 adjusted at 25 °C). 2 mg of BSA/ml was added.

Reagents

All reagents were purchased from Sigma, except ATP and ADP, which were obtained from Boehringer.

Analysis of the experimental results

The values in Tables and Figures are expressed as means ± S.E.M. The apparent K_m for ADP or ATP was estimated from a linear regression of double-reciprocal plots or by non-linear least-squares fit (Enzfitter). Statistical comparisons were made using the ANOVA test (variance analysis and Fisher test), and $P < 0.05$ was taken as the level of significance.

RESULTS

Complete release of cytoplasmic components from cardiac cells by saponin

The selective permeabilization of the cell membrane by saponin or by digitonin is based on the high affinity of these agents for cholesterol and significant differences of the cholesterol contents in the cellular or subcellular membranes [14,23]. The surface membrane of the muscle cells, the sarcolemma, contains the highest amount of cholesterol, and is therefore dissolved within minutes in the presence of saponin or digitonin in concentrations of 50–100 μg/ml, while the digitonin concentration should be increased about 100 times, up to 6 mg/ml, to permeabilize the mitochondrial outer membrane [14]. Figure 1(A) shows that, under conditions of such a selective permeabilization of sarcolemma, all the LDH present in the cells is rapidly released into the medium. The same was observed for creatine + phosphocreatine (total creatine, Figure 1B). In both cases, dissolution of mitochondrial membranes by Triton X-100 gives no additional release of either LDH or total creatine. These results show complete permeabilization of sarcolemma by saponin in the concentration used and loss of its barrier function for low-molecular-mass metabolites and even for macromolecules. Because of complete loss of the barrier function of sarcolemma, these preparations are usually called 'skinned fibres' since the name 'permeabilized cells' is often also used in the case of only partial loss of this barrier function (only of low-molecular-mass metabolites). At the same time, all functional properties of mitochondria in skinned cells are completely intact. The acceptor control ratio of respiration by ADP (2 mM) is close to 10 (in the presence of Mg^{2+}), showing a high degree of coupling of respiration and oxidative phosphorylation at the mitochondrial inner membrane, and in the KCl medium exogenous cytochrome *c* has no effect on respiration rate, showing the intactness of mitochondrial outer membrane and its integrity [14]. Altschuld et al. [24] has shown previously that, in skinned cardiomyocytes, mitochondria are preserved well and even have ion gradients across their membranes.

Figure 1(C) shows the confocal image of the mitochondria in the skinned cardiac fibres as revealed by MitoTracker®, a fluorescent dye dependent on the membrane potential. The dye is taken up in the presence of ATP or of substrates and oxygen, and then fixes itself covalently at the thiol groups in the mitochondrial matrix; thus, its uptake is irreversible [25]. Figure 1(C) shows that all mitochondria are arranged into parallel

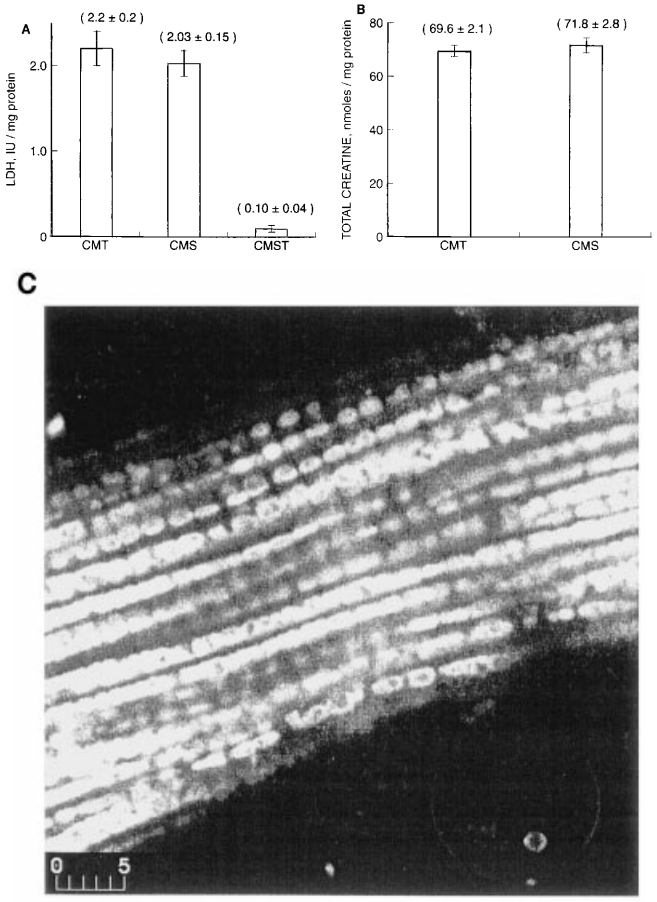


Figure 1 Characterization of permeabilization procedure and skinned cardiac fibres

(**A**) Total extraction of LDH activity from isolated cardiomyocytes after 30 min of treatment with saponin (50 μg/ml). (**B**) Complete extraction of total pool of creatine from isolated cardiomyocytes after 30 min of treatment with saponin (50 μg/ml). Mean $\pm$ S.D. are given for five experiments. CMT, supernatant obtained after treatment of cardiomyocytes with Triton X-100 (2%) and centrifugation for 5 min at 1000 ***g***. This supernatant contains all the cellular pools of metabolites and enzymes (including those in mitochondrial matrix) which are not strongly fixed to myofibrils or cytoskeleton. CMS, supernatant obtained after treatment of cardiomyocytes for 30 min with saponin (50 μg/ml) to selectively permeabilize the sarcolemma, and by subsequent centrifugation for 5 min at 700 ***g***. This fraction contains metabolites and enzymes liberated after permeabilization of sarcolemma and thus localized in the cells in the bulk water phase in the cytoplasm. CMST, supernatant obtained after Triton X-100 (2%, v/v) treatment of the pellet obtained after saponin treatment of cardiomyocytes and centrifugation (step CMS). This fraction contains metabolites and enzymes which may be localized in mitochondria or in the cells not permeabilized by saponin (if that was not effective). (**C**) Confocal imaging of mitochondria in skinned cardiac fibres. The mitochondrial matrix was loaded with potential-sensitive fluorescent dye, Mitotracker® (see the Experimental section). The loading was performed in solution A, containing ATP and phosphocreatine. Homogenous labelling of all mitochondrial populations in rat heart skinned fibres is seen. Note the 'mitochondrial columns' between the myofibrils and the very regular arrangement of mitochondria within the cell. The scale bar represents 5 μm.

arrays between myofibrils in a very regular way. There are apparently no heterogeneous subpopulations of mitochondria in these cells. Previously we have seen that extraction of myosin, by 800 mM KCl solution, to produce ghost cardiomyocytes, does not change the mitochondrial position in the cells [15]. Thus the specific and precise intracellular organization of mitochondria seems to be mostly due to their interaction with cytoskeleton, probably mediated by some binding protein [14,15,26].

Kinetics of regulation of mitochondrial respiration by endogenous ADP versus exogenous ADP

In the skinned muscle cells the ATPases in myofibrils and in the sarcoplasmic reticulum are intact [14,24,27], and therefore these preparations are suitable for studies of the regulation of mitochondrial respiration *in situ* by endogenous ADP produced by intracellular ATPases during hydrolysis of exogenous ATP. The kinetics of this type of regulation of respiration is shown in Figure 2. We compared the kinetics of regulation of mitochondrial respiration *in situ* in two types of cells; in skinned fibres from rat heart and soleus muscle, and in their ghost or phantom fibres after extraction of a significant part of the myosin by 800 mM KCl [14,15]. In skinned fibres the source of ADP is MgATPase of both the myofibrils and the sacroplasmic reticulum, while in ghost fibres the relative role of the latter is obviously more important, and due to removal of a significant part of myofibrillar proteins the diffusion of ADP or ATP could be easier (if sarcomeric structures somehow restrict the diffusion). When mitochondrial respiration rate is plotted against exogenous ATP concentration in the medium, the hyperbolic dependence is seen for both skinned fibres and ghost fibres (Figure 2A). It gives a straight line in double-reciprocal plots (Figure 2B) and an apparent K_m value equal to 300 ± 23 μM for skinned cardiac fibres (Figure 2B) and for ghost fibres from the heart. Alteration of the Ca^{2+} concentration changes the V_{max} of respiration but practically does not change the apparent K_m value for exogenous ATP (Figure 2B). Under conditions of regulation of respiration by endogenously generated ADP, the concentration of ADP in the medium was measured chromatographically. An increase in both exogenous ATP and Ca^{2+} concentration resulted in elevation of the ADP concentration in the medium up to 20 μM (Figure 2C). In the presence of ghost fibres the ADP concentration in the medium with Ca^{2+} was lower than that in the case of skinned fibres because of loss of myosin. From data presented in Figures 2(A) and 2(C), the relationship between the respiration rate and ADP concentration in the medium can be found (Figure 2D). This relationship produces a straight line for skinned cardiac fibres, the higher ADP concentrations and higher respiration rates corresponding to higher Ca^{2+} concentrations. This fits with the conclusion of Khuchua et al. [8] and Fiolet et al. [28] that the mitochondrial respiration in skinned fibres is regulated by Ca^{2+} via alteration of the Ca, MgATPase activities of the myofibrils and sarcoplasmic reticulum. The values of respiration rates for given ADP concentrations in the medium are higher for ghost fibres than for skinned fibres, and the relationship is no longer linear (Figure 2D).

When ATP was not added but respiration was initiated by exogenous ADP, the latter was added to skinned or ghost fibres at concentrations up to 2–3 mM (Figure 3) to achieve maximal activation of respiration in accordance with many earlier observations [12–16,26,27]. Interestingly, the apparent K_m values for both exogenous ADP and exogenous ATP in regulation of mitochondrial respiration are very close and are, in fact, practically the same (compare Figures 2B and 3). One may conclude from these data that it is the total concentration of exogenous adenine nucleotides, ADP plus ATP, which is the most important parameter in regulation of respiration in skinned cardiac fibres *in situ*.

Possible influence of the diffusion of ADP in the bulk-phase of intracellular water

To understand and analyse the dependence of the relationship between respiration rate and ADP (or ATP) concentration in the medium upon the source of this substrate, one needs to know if

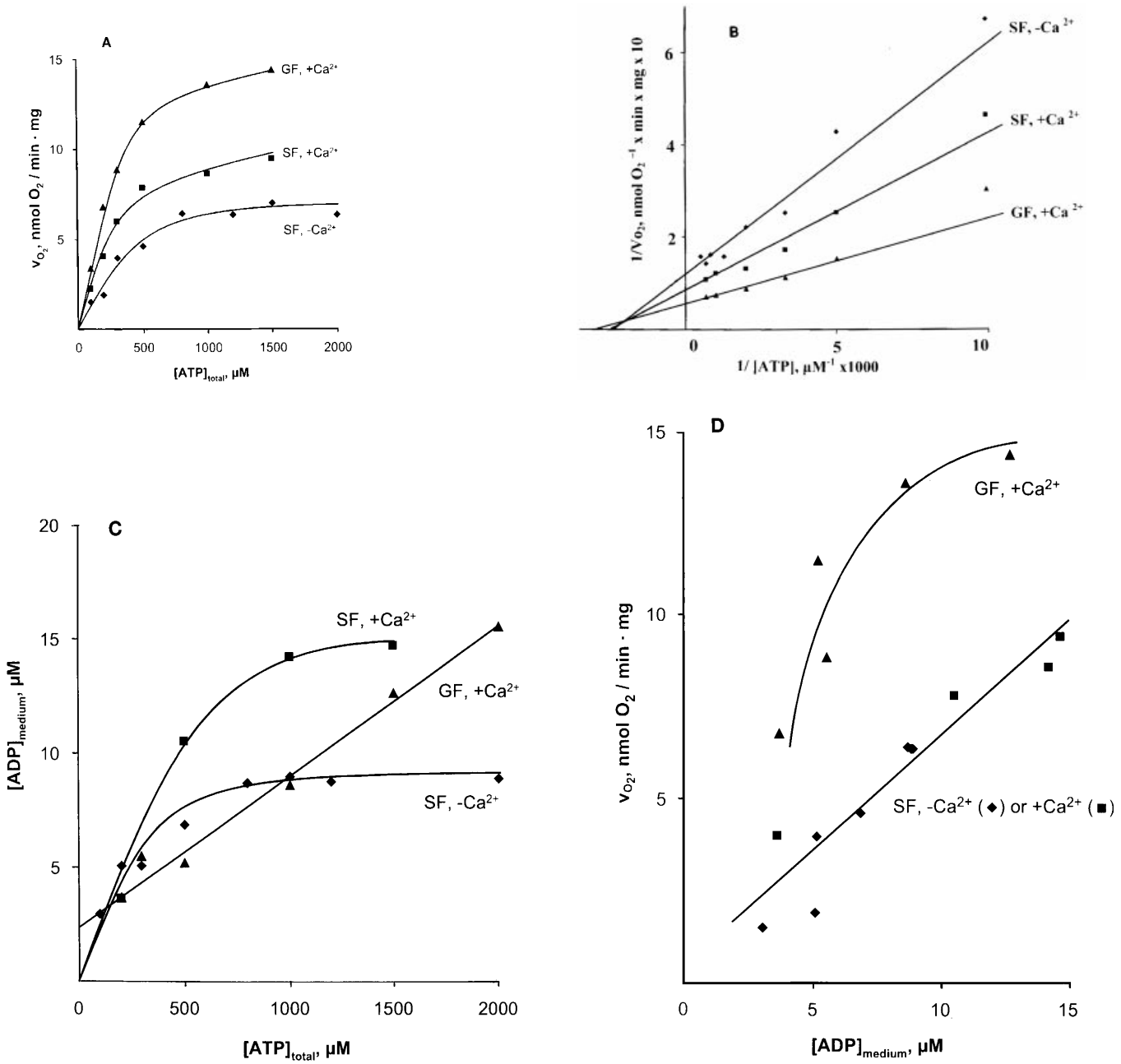


Figure 2 Kinetics of the regulation of mitochondrial respiration in skinned rat cardiac fibres by endogenous ADP

(**A**) Respiration rates in skinned fibres (SF) and ghost fibres (GF) in sucrose solution without calcium ($-CA^{2+}$) and in normal solution B with calcium ($+Ca^{2+}$). Each point corresponds to the initial rate of respiration of a single fibre preparation at the specified exogenous ATP concentration. The curves are representatives of one experiment of between five to ten. Analysis of all data by the non-linear least-squares fit gave: $K_m = 327 \pm 46$ μM, $V_m = 18.0 \pm 1.0$ nmol of O/min per mg (GF, $+Ca^{2+}$); $K_m = 320 \pm 36$ μM, $V_m = 11.8 \pm 0.9$ nmol of O/min per mg (SF, $+Ca^{2+}$); and $K_m = 356 \pm 58$ μM, $V_m = 8.6 \pm 0.9$ nmol of O/min per mg (SF, $-Ca^{2+}$). (**B**) Linearization of data from Figure 2A in double-reciprocal plots. (**C**) ADP concentration in the medium measured for any ATP concentration shown in Figure 2(A). The curves show one representative experiment of three. Analysis of all data by the non-linear least-squares fit gave the 'half-saturating' external [ATP] values 363 ± 84 μM (SF, $+Ca^{2+}$) and 269 ± 46 μM (SF, $-Ca^{2+}$) and the corresponding maximal [ADP] in solution 19.9 ± 1.5 μM and 10.9 ± 0.6 μM. (**D**) Respiration rates versus ADP concentration in the medium measured by HPLC. Data are taken from Figures 2(A) and 2(C).

the ADP in the intracellular bulk-water phase can equilibrate sufficiently rapidly with surrounding medium. Two approaches have been used to solve this problem. The first approach is to use the Einstein–Smoluchowski equation for Brownian movement in one dimension [29]:

$$D = x^2/2t$$

where D is diffusion coefficient and x^2 is mean-square displacement for the transit time, t (to describe diffusion of ADP molecules in three dimensions, the equation will be $D = x^2/6t$; [29] and the Discussion section). From this equation one can calculate the time needed for ADP or ATP to diffuse from or to the mitochondria through the sarcomere space in myofibrils or from inside of cardiomyocytes into the medium. Some of the

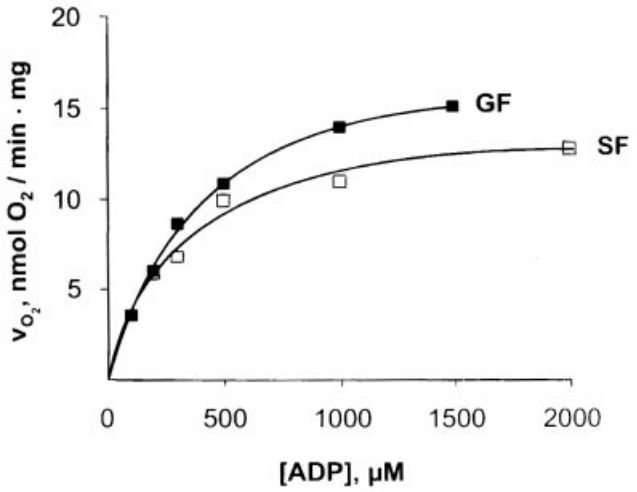


Figure 3 Respiration rates of skinned cardiac fibres (SF) and ghost fibres (GF) in dependence of the exogenous ADP concentration

One representative experiment of 15 is shown. Analysis of all data by the non-linear least-squares fit gave $K_m = 320 \pm 36$ μM, $V_m = 14.9 \pm 0.5$ nmol of O/min per mg (SF) and $K_m = 354 \pm 39$ μM, $V_m = 17.4 \pm 0.7$ nmol of O/min per mg (GF).

Table 1 Calculation of the time of displacement of ADP or ATP between bulk water phase in cardiomyocytes and medium, and ADP/ATP turnover time in mitochondria *in situ* at 20 °C

For the calculations, the value of diffusion constant for ADP (and ATP) $D = 0.33 \times 10^{-9}$ m$^2 \cdot$ s^{-1} $= 330$ μm$^2 \cdot$ s^{-1} measured by Graaf et al. at 20 °C were used [53]. The calculation of displacement time, t, was carried out according to the Einstein–Smoluchowski's equation for one dimension ($D = x^2/2t$ [29]). Turnover time for ADP in mitochondria *in situ*, τ, time of its phosphorylation in the coupled reactions in mitochondria by the oxidative phosphorylation complex (F_1 F_o-ATPase coupled to the respiratory chain and adenine nucleotide translocator), was found from the catalytic-centre activity ('turnover number') of cytochrome oxidase and the P/O ratio. The following values of parameters were used: maximal oxygen consumption rate (at 20–22 °C) in the range of 20–30 nmol of O_2/min per mg dry weight or 4–6 nmol/min per mg wet weight of tissue (Figure 2, [13–16]), the cytochrome aa_3 content (cytochrome oxidase) 1 nmol/mg of protein in heart mitochondria [54], the mitochondrial content in heart tissue between 60–90 mg of mitochondrial protein in g of wet tissue [55], and P/O ratio equal to 3.

Displacement (μm)	Time (t) (ms)
1.0 (sarcomere diameter)	1.5
5.0 (half-radius of cardiomyocyte)	38
10 (from centre out of cell)	151.5
Turnover time of ADP/ATP in mitochondria (τ) ...	130–200

results of these calculations are given in Table 1 for the isolated ghost cardiomyocyte with average diameter of 20 μm. Because of the removal of cytoplasmic proteins and the significant part of myosin, it is justifiable to assume that the D of ADP and ATP in the bulk phase inside the cells does not differ from that in water solutions. In this case, the important thing to know is how rapidly ADP can be exchanged between extracellular medium and intracellular bulk phase in comparison with the rate of ADP phosphorylation in mitochondria (ATP–ADP turnover rate). These simple calculations show that, to traverse the sarcomere space with diameter of 1 μm, ADP or ATP need only 1.5 ms. Since mitochondria are arranged regularly between myofibrils in the plane of fibres' cross-section, the time of interest is that which is necessary for ADP or ATP to traverse the distance equal to

Table 2 Apparent K_m values for exogenous ADP in solution B (with calcium) and in iso-osmotic solution of sucrose (without calcium, and with or without magnesium)

Medium	K_m [ADP] (μM)
Solution B ($+Ca^{2+}$, $+Mg^{2+}$)	320 ± 36
Sucrose (240 mM) solution ($+Mg^{2+}$, $-Ca^{2+}$)	340 ± 50
Sucrose (240 mM) solution ($-Mg^{2+}$, $-Ca^{2+}$)	306 ± 16

half the radius of cardiomyocytes, 5 μm, since this could be taken as an average time of exchange between mitochondria and external medium. This time is 38 ms (Table 1). To diffuse through the water from outside into the cell centre, ADP needs about 150 ms. These diffusion times should be compared with the time needed to rephosphorylate ADP into ATP in mitochondria (turnover time). This time is between 100–200 ms at 20 °C. Thus ADP diffuses more rapidly in the space occupied by the cardiomyocytes than it is utilized, provided that its diffusion coefficient is that found is aqueous solution (Table 1).

The second approach is to use the mathematical reaction–diffusion model of energy transfer to study the influence of diffusion on the profile of metabolites within the sarcomere space. This was done recently [30], and the results showed that, even if the D for ADP was increased 10^5 times from its value in water, the ADP profile did not change. Thus diffusion of ADP in the bulk-water phase in cells is already very rapid.

A similar conclusion can be made from experiments in which a respiratory-control parameter, apparent K_m for exogenous ADP, was measured in both solution B (with Ca^{2+}) and sucrose solution (without Ca^{2+} and with or without Mg^{2+}). In this case, significant activation of ADP regeneration from endogenous ATP by Mg^{2+} and Ca^{2+} (see Figure 2) had no effect on the value of the apparent K_m for exogenous ADP (Table 2). Thus the apparent K_m for exogenous ADP does not depend on the Ca,Mg ATPase activity, which is very low in the sucrose medium without Ca^{2+} and Mg^{2+} (in our experiments it did not exceed 15–20 % of V_{max} in solution B), but high in the solution B with both cations present (see below and [15]). This again means that the diffusion of exogenous ADP into fibres is sufficiently rapid, in comparison with the rates of ADP turnover, and does not contribute to high K_m values for this substrate.

In conclusion, all these results are consistent with the view that ADP or ATP diffusion in the bulk-water phase is sufficiently rapid, compared with the respiration and ATPase reaction rates. In this case the differences in kinetics of regulation of mitochondrial respiration by ADP, depending on the source of ADP described above, show that most probably ADP produced in intracellular ATPase reactions is generated in the intracellular compartments with locally restricted diffusion (D is locally lower than assumed) and/or endogenous ADP is directly channelled to mitochondria before its release into the medium.

Effects of exogenous ADP-trapping system and creatine: evidence for direct metabolic channelling of ADP

This explanation can easily be tested by using an exogenous ADP-trapping system consisting of PK and PEP, which competes with mitochondria for the ADP [31]. The results of these experiments are shown in Figure 4. First, we tested the efficiency of the PK + PEP system used in experiments with isolated heart mitochondria, which have low MgATPase activity (Figure 4). Figure 4(A) shows that addition of ADP in concentrations up to 3 mM (as used in experiments with skinned fibres) activates

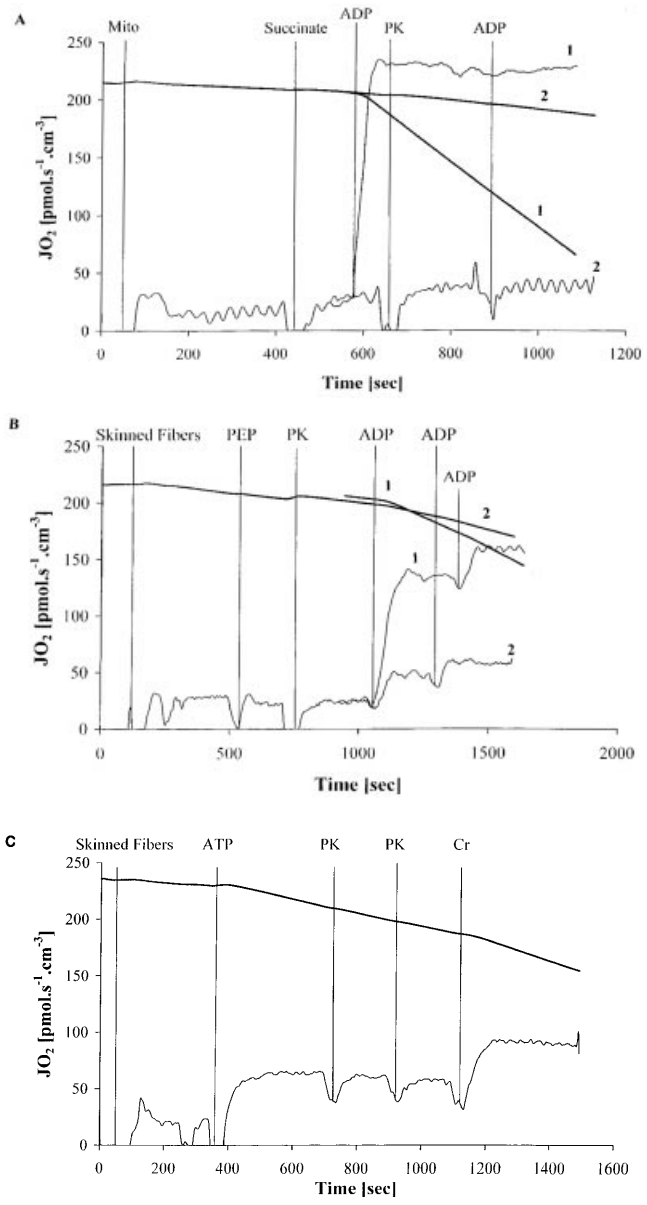


Figure 4 Effects of the PEP + PK system on respiration in isolated heart mitochondria, skinned cardiac fibres and skinned soleus-muscle fibres

(**A**) Isolated heart mitochondria were activated by addition of exogenous ADP (3 mM). (**B**) Skinned cardiac fibres in sucrose medium without Ca^{2+} were activated by successive additions of exogenous ADP (0.5 mM and 1 mM). (**C**) Skinned rat m. soleus fibres were activated by endogenous ADP produced inside the fibres from ATP (2 mM). Two recordings are shown: the change of oxygen concentration with time (upper traces) and its first derivation (lower traces), showing directly the rate of oxygen consumption. Isolated mitochondria (Mito) were introduced into the respiratory chamber to concentration of 75 μg/ml. In all cases, the reaction medium contained PEP (3 mM) and glutamate (5 mM) and malate (2 mM) as respiratory substrates. Skinned fibres were added at approx. 2–3 mg wet weight/2 ml. Representative recordings of four reproducible experiments are given. (**A**) and (**B**) Recordings 1 are in the absence of PK, and in recordings 2 PK was added to give an activity of 10 units/ml before ADP. (**C**) Oxygraphic recordings of the effect of the exogenous ADP-trapping system of PK and PEP on the respiration of skinned fibres from rat m. soleus induced by exogenous ATP. Respiration in the skinned fibres was initiated by addition of ATP (2 mM) in the presence of PEP (5 mM). Then PK (20 units) was added twice and finally creatine (20 mM) was added.

respiration of isolated rat heart mitochondria by a factor of 8–10. In experiments with isolated mitochondria there is no diffusion barrier for ADP and after ADP addition State 3 respiration is established within about 1 min due to slow response time of the very stable Oroboros electrode system used. When PK was added (PEP was already in the medium) to give a final activity of 10 units/ml, all ADP added was used up within seconds and State 3 respiration was not observed (Figure 4A). The PK + PEP system was equally effective in eliminating exogenous ADP when the skinned cardiac fibres were used in Ca^{2+}-free sucrose medium to avoid endogenous ADP production in the Ca,Mg ATPase reactions (Figure 4B). Activation of mitochondrial respiration in fibres under those conditions by successive addition of 0.5 mM and 1.0 mM exogenous ADP was characterized by the acceptor control ratio of mitochondria respiration ratio 6–7 without PK and PEP and by the same electrode response time as with isolated mitochondria (Figure 4B). When PK and PEP were added before ADP, only State 4 respiration was observed due to very rapid phosphorylation of ADP into ATP (Figure 4B).

However, very different results were obtained in experiments with skinned muscle fibres in solution B, when endogenous ADP was produced in the Ca,Mg ATPase reactions. The addition of exogenous ATP, in final concentration of 2 mM, activated respiration about 4–5-fold. A powerful ADP-trapping system (PK + PEP) decreased the rate of respiration initiated by the addition of 2 mM ATP not more than 20 % (Figure 4C). In these experiments the total activity of added PK (20 units/ml) exceeded the combined rates of the ATPase and oxidative phosphorylation in oxygraphic cells (maximally 0.2 μmol/min per ml) by two orders of magnitude. One may think, however, that this activity of PK is relatively low, considering the local ATPase activities inside the intracellular space of fibres. The ATPase activity of skinned cardiac fibres is of the order of 10 units/g of wet weight (see below), and as 1 g of wet weight contains 0.6 ml of intracellular water [11], that gives 16 units/ml for ATPase locally in the intracellular water space, which is of the same order or even lower than added PK. Further addition of PK had no effect on the respiration rate. Thus, the results shown in Figure 4(C) demonstrate that endogenously generated ADP is not easily accessible for PK if mitochondrial respiration is activated; instead the latter process has a preferable access to endogenous ADP. That means that ADP is directly channelled from the endogenous ATPase to mitochondria.

However, in the presence of 2 mM ATP and the exogenous PK + PEP system, addition of creatine gives a strong increase in the respiration rate (Figure 4C). In our recent study we have shown that these stimulating effects of creatine are observed at very low and constant ADP concentrations in the medium and not observed if the mitochondrial creatine kinase is knocked out by genetic manipulations [32]. This shows the importance of the creatine kinase system in the facilitated diffusion of ADP and in the regulation of respiration.

Similar results were obtained for the skinned cardiac fibres (Figure 5). Mitochondrial respiration *in situ*, maintained by endogenous ADP produced in the rat heart skinned fibres, in the presence of 2 mM MgATP, was inhibited not more than 40 % by PK + PEP, and increasing the activity of PK from 10–30 units/ml did not result in any further changes of respiration rate. In the presence of creatine (20 mM), when ADP was also generated inside the intermembrane space of mitochondria, the PK + PEP system had almost no effect on respiration maintained by endogenous ADP. Interestingly, when the skinned fibres were treated with a low concentration of trypsin, the efficiency of inhibition of respiration by the PK + PEP system (the availability of endogenous ADP for the PK), increased significantly, but the addition of creatine still increased respiration (Figure 5) by activation of ADP production in the mitochondrial intermembrane space. The effects of trypsin are explained by its

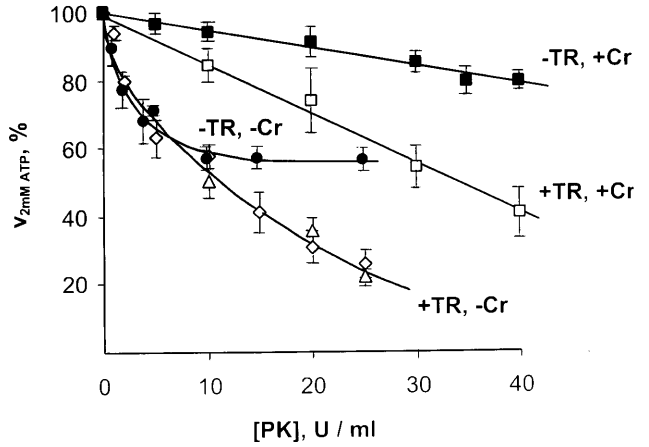


Figure 5 Effects of increasing PK activity in the medium on the rate of respiration of skinned cardiac fibres maintained by endogenous ADP production

Experiments were carried out in the presence of 2 mM MgATP and in the absence of creatine (−Cr) or in the presence of 20 mM creatine (+Cr) before treatment with trypsin (−TR) and after treatment with trypsin (+TR) for 5 min (0.1 μM, squares and diamonds; 0.5 μM, triangles) in solution B without BSA and then washing the fibres in solution B containing BSA. The concentration of active trypsin in stock solution was determined by titration with *p*-nitrophenyl quanidinobenzoate by a standard procedure [56]. Results are means ± S.D. for three to five experiments.

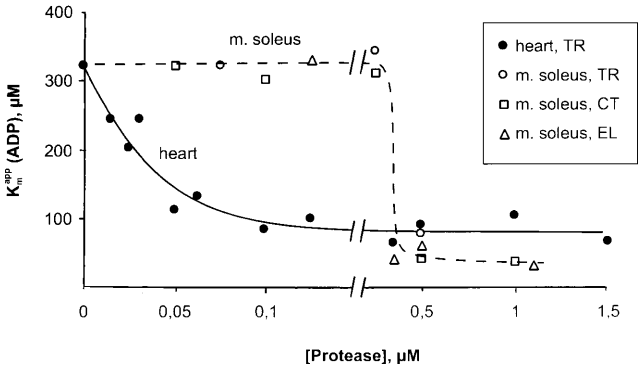


Figure 6 Effects of proteases on skinned fibres from rat heart (continuous lines) and soleus muscle ('m. soleus') (broken line) on the apparent K_m for exogenous ADP

K_m values correspond to the mean value obtained from three or four curves. In all experiments, S.D. values were in the range 15–41 μM. The method of pretreatment of the fibres by proteases before the kinetic experiments was carried out as described in Figure 5. Abbreviations: TR, trypsin; CT, chymotrypsin; El, elastase.

ability to cause the disorganization of cellular structures, as described below.

Effects of limited proteolysis on kinetic parameters of respiration regulation enzyme activities and cell structure

Figure 6 shows the changes in apparent K_m for exogenous ADP in the regulation of respiration in mitochondria in skinned fibres from rat heart and soleus muscle induced by a short incubation (5 min) with increasing amounts of protease (trypsin, chymotrypsin or elastase). The changes in apparent K_m for ADP are different for these two types of fibres, but finally, at a protease concentration of 500 nM the apparent K_m for ADP is decreased

Table 3 Decrease in the apparent affinity of mitochondrial Mg-ATPases, from skinned cardiac fibres, for MgATP after brief periods of treatment with trypsin at different concentrations

[Trypsin] (nM)	Apparent K_m [MgATP] (μM)
0	300 ± 23
10	211 ± 18
50	123 ± 25
200	116 ± 20
500	77 ± 17

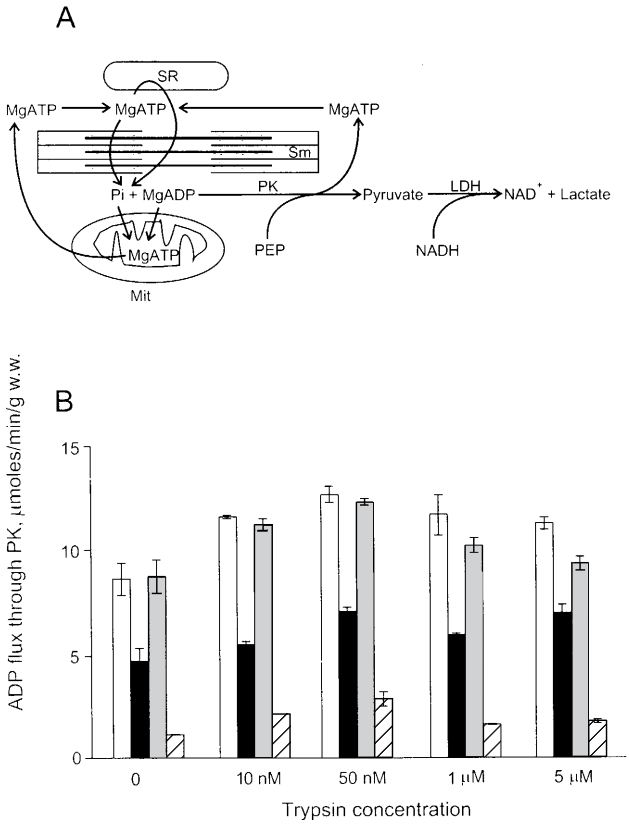


Figure 7 Analysis of competition between mitochondria and the exogenous PK + PEP system for ADP generated in ATPase reactions

(**A**) A scheme for the ATPase reactions coupled either to mitochondrial oxidative phosphorylation or to extramitochondrially added PK + PEP system of ADP rephosphorylation. (**B**) Changes of the rate of release of ADP from cardiac muscle fibres and its flux through the PK + PEP system by activating or inhibiting the mitochondrial oxidative phosphorylation after treatment of fibres with trypsin (10 nM–5 μM, for 5 min at 4 °C). White columns, ATPase reaction rate (expressed as ADP flux through PK) in the absence of oxidative phosphorylation. Black columns, ADP flux through PK after switching on oxidative phosphorylation by addition of the substrates, 10 mM glutamate and 2 mM malate. Grey columns, ADP flux through the PK + PEP system in the presence of 98 μM atractyloside which abolishes oxidative phosphorylation due to inhibition of adenine nucleotide translocase. The difference between the ATPase activities with and without glutamate and malate equals the mitochondrial flux of ADP that is not accessible to the PK + PEP system. Striped columns, ATPase activities in the incubation medium after removal of the fibres by filtration of the medium through 0.2 μm syringe filter. Results are means ± S.D. for two to five experiments.

in both cases to the values of 40–60 μM. This is in accordance with our previous observations [12–16,32]. A similar effect was seen for the apparent K_m for exogenous ATP (Table 3). No significant changes were observed in ADP fluxes measured

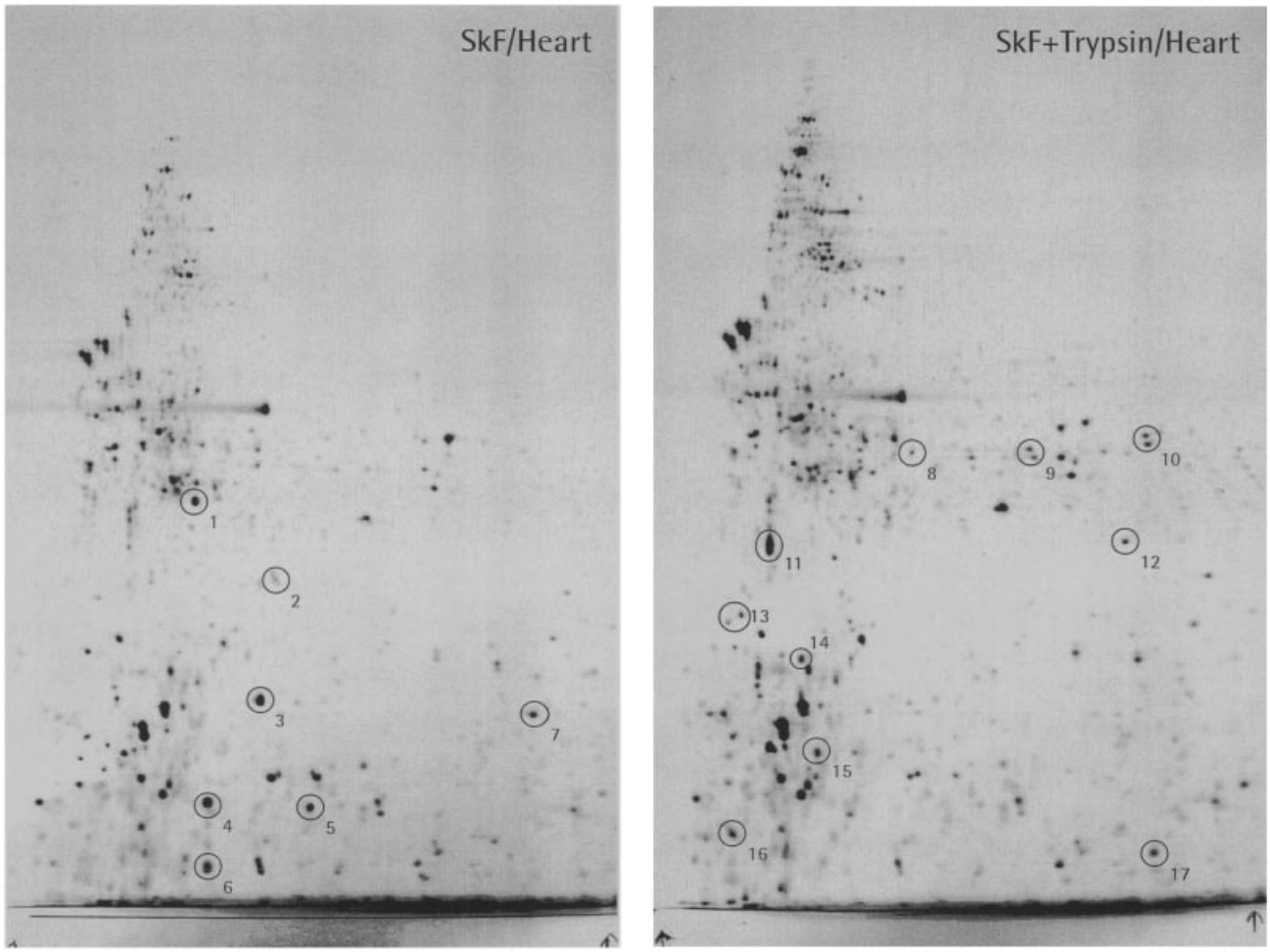


Figure 8 Two-dimensional electrophoregrams showing rat heart skinned fibre protein composition before and after treatment with 2.5 μM trypsin for 15 min

(Left panel) Circles identify the spots which disappeared after trypsin treatment. Other spots were not significantly changed. (Right panel) Circles identify the spots which appeared after trypsin treatment (products of degradation of some proteins).

directly by a spectrophotometric method using PK + PEP, LDH and NADH (Figure 7). In this coupled system, the rate of oxidation of NADH is equivalent to the rate of ADP release into the medium (Figure 7A). Addition of exogenous ATP resulted in a decrease in NADH with the stable-steady-state rate corresponding to the release of ADP (Figure 7B). In the absence of respiration, this is the rate of the Mg^{2+}-ATPase reaction. When the mitochondrial-respiratory substrates glutamate and malate were added, the rate of NADH oxidation decreased by about 50 % (Figure 7B); however, when the mitochondrial uptake of ADP was inhibited by atractyloside, the rate of oxidation of NADH was restored to its initial value (Figure 7B). These results give further support to the hypothesis that ADP, generated by intracellular MgATPases, is first available for mitochondria before its release into the medium. None of these fluxes were changed significantly by the proteolytic treatment (Figure 7) within the range of the protease concentrations (higher than 10 nM) which caused significant change in apparent K_m values for both exogenous ADP and ATP. The maximal respiration rate measured in the presence of 2 mM ADP was not changed under these conditions (results not shown). This confirms our previous observation that the proteases (up to 5 μM in concentration) did not effect the isolated mitochondria *in vitro* [13].

To understand what kind of changes in protein composition of fibres are induced by the proteolytic treatment used in this work, we performed two-dimensional electrophoretic analysis of the skinned fibres before and after treatment with trypsin (Figure 8). In general, the changes induced are very specific. Figure 8 (left panel) shows the protein composition of skinned fibres from rat heart before the treatment with trypsin, and Figure 8 (right panel) shows it after the treatment with trypsin (15 min, 5 μM). Seven spots corresponding to proteins which disappear after proteolytic treatment (Figure 8, right panel) are shown in Figure 8 (left panel). The other spots corresponding to stable proteins were almost unchanged. The 17 spots shown by circles in Figure 8 (right panel) show the peptide fragments which are evidently the results of proteolysis of some larger proteins. Thus the treatment used is rather specific and mostly concerns seven very sensitive proteins. There are interesting targets for studies of possible candidates for proteins participating in the control of mitochondrial position and function in these cells.

The explanation of the observed phenomena described above, the rapid increase of the apparent affinities for exogenous ATP and ADP (Figure 6 and Table 3) and the increase in accessibility of the endogenous ADP for the PK + PEP system after short-time-selective-proteolytic treatment of skinned fibres (Figure 5)

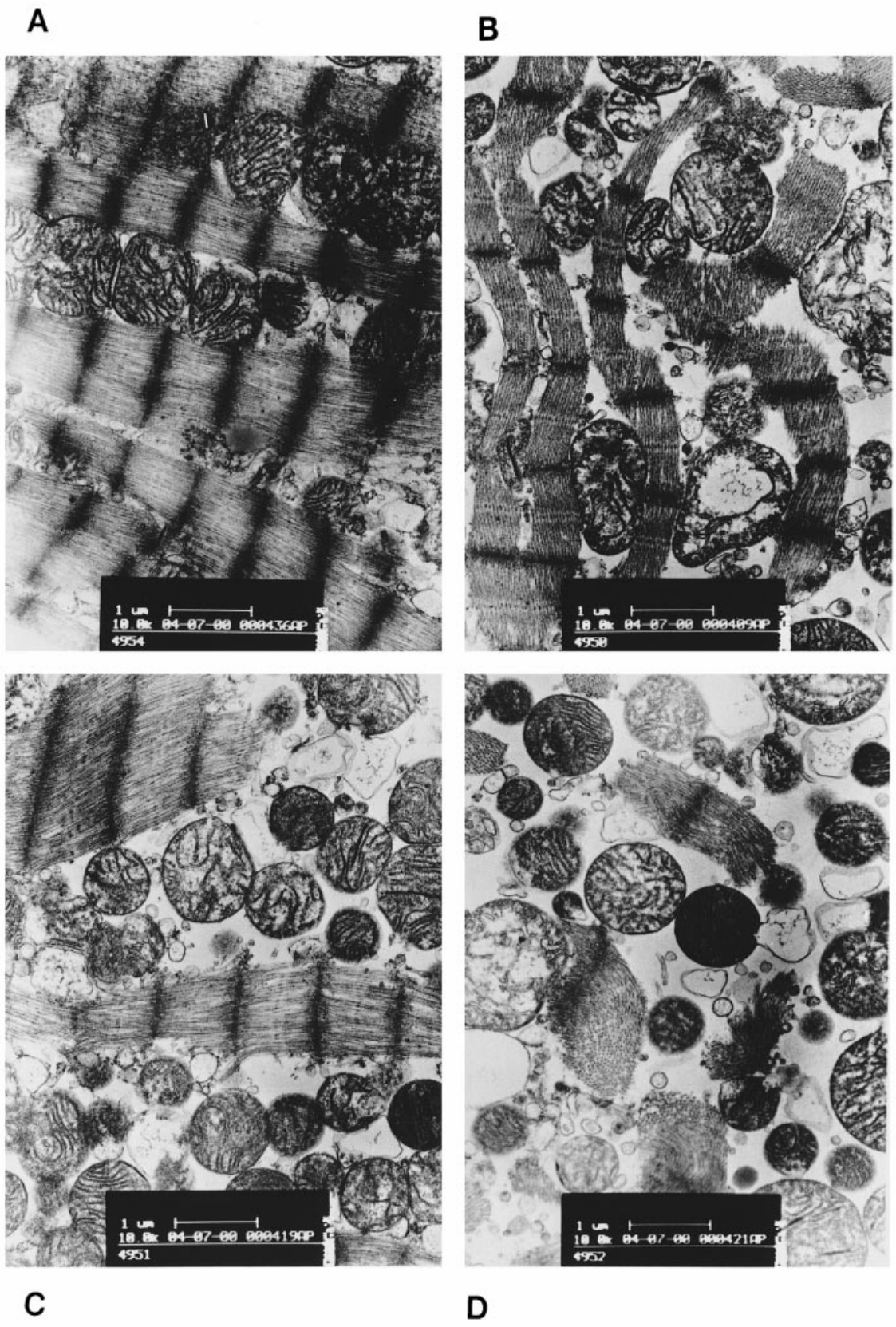


Figure 9 Electron microscopy of skinned cardiac fibres: effects of treatment with trypsin at different concentrations on the cell structure

Fibres were treated before the fixation and observation procedure (see the Experimental section) with the trypsin in the concentrations indicated below in solution B for 5 min as described in Figure 6. A, control (no treatment); B, 50 nM trypsin; C, 1 μM trypsin; D, 5 μM trypsin.

when no changes in enzyme activities are observed (no inactivation of enzymes involved; Figure 7), is given in Figure 9. The explanation is the disorganization of the structure of the muscle cell. Figure 9 shows the results of an electron-microscopic investigation of cardiac muscle fibres. In intact (non-treated) fibres mitochondria are regularly arranged between myofibrils (see also Figure 1C), mostly at the level of the A-band in close contact with the sarcomeres and sarcoplasmic reticulum (Figure 9A). A 5 min treatment with 50 nM trypsin (Figure 9B) or 1 μM trypsin (Figure 9C), which already decreases the apparent K_m for exogenous ADP (and ATP) (Figure 6) but does not change the Mg-ATPase activities and ADP fluxes (Figure 7), very clearly detach mitochondria from sarcomeres and also from sarcoplasmic-reticulum vesicles and disorganize the structural organization of sarcomere. Previously, Higuchi [33] found that this disorganization of the sarcomere structure by low concentrations of trypsin is due to degradation of titin filaments. After treatment with 5 μM trypsin, the cell structure is completely disorganized and the cell interior seems to be a homogeneous mixture of mitochondria and fragments of sarcomeres, which still somehow manage to stay static inside the cells (Figure 9D). In this mixture, the properties of mitochondria (apparent K_m for ADP) or myofibrils (apparent K_m for ATP) are close to those observed *in vitro* after their isolation [26]. Evidently, the kinetics of the regulation of mitochondria *in situ* in muscle cells is influenced by cell organization.

DISCUSSION

The results of the present study show that, in oxidative red muscle (heart and soleus muscle) cells, the kinetics of regulation of mitochondrial function are closely linked to the structural organization of these cells. These results show that mitochondria, consuming ADP and producing ATP, behave as if they are integrated (probably via interaction with proteins associated with the cytoskeleton and responsible for mitochondrial distribution in the cells) into functional complexes with adjacent ADP-producing systems; the Mg-ATPases of myofibrils and the Ca,Mg-ATPases of the sarcoplasmic reticulum. ADP produced within these units, or complexes, does not equilibrate easily with ADP in the bulk-water phase. In the case of permeabilized cells the bulk phase is a medium, whereas in the cells *in vivo* it is probably the cytoplasm. Moreover, within these functional units, the energy is transferred mostly via enzyme networks composed of the creatine kinase and adenylate kinase systems. For convenience, these functional complexes may be called ICEUs (Scheme 1).

These results and conclusions are consistent with ultrastructural studies showing intracellular localization of mitochondria in muscle cells by Ogata and Yamasaki [34]. Ogata and Yamasaki [34] used glutaraldehyde-fixed muscles which were frozen, fractured and macerated by the aldehyde–osmium–DMSO–osmium procedure to remove the cytoplasmic matrix and myofilaments from the fractured surface and to expose large areas of mitochondria and the sarcoplasmic reticulum. Observation of these samples by high-resolution scanning electron microscopy showed that, in oxidative muscle fibres, mitochondria are organized into three-dimensional structures or networks where, in the intermyofibrillar space, multiple mitochondria form thick mitochondrial columns at the A-band level, while in the white (fast-twitch) muscles mitochondria were localized only at the I-band level near the Z-line [41]. Interestingly, this different localization correlates with the differences in the kinetics of the regulation of respiration, the apparent K_m for ADP is two orders of magnitude lower in the fast-twitch (glycolytic) than in slow-twitch (oxidative) muscle fibres [16]. In cardiomyocytes and in 'ghost' cardiac cells mitochondria are regularly localized between myofibrils at the level of the A-band (see Figure 1C and [15]). An alteration of the cardiac-cell cytoskeleton by knock-out of the desmin gene changes the mitochondrial localization in cardiac cells and significantly decreases the apparent K_m for ADP (increases the affinity for ADP) [15,35]. In the present study we show that precise localization of mitochondria between myofibrils can also be changed by short incubation of skinned cardiac cells with proteases, and that this treatment changes the kinetics of the regulation of respiration by exogenous ADP, without any changes in the activities of enzymes involved.

Striking differences in the relationship between ADP concentration in the medium and respiration rate of mitochondria *in situ* in skinned fibres, in their dependence upon the source of ADP, were first described by Kummel [36]. We have studied in detail the kinetics of the regulation of respiration by exogenous ADP in the skinned muscle cells and in hepatocytes (reviewed in [13,26]). Both the present-study and that of Kummel [36] found high values for the apparent K_m for exogenous ADP in the regulation of respiration in permeabilized isolated cardiomyocytes, which exceeded the value of this parameter for mitochondria *in vitro* by an order of magnitude. In addition, Kummel found that the apparent K_m for ADP was lower when ADP was produced in the Ca,Mg-ATPase reaction [36]. This observation is confirmed in the present study.

For interpretation of these observations, the critical issue is whether they can be trivially explained by limited diffusion of ADP (and ATP) from medium into the fibre (or isolated cell) simply because of the cell size and geometry (this may be called 'non-specific diffusion limitation'). All experimental and theoretical results gathered until now give us the following five clear and strong arguments against this simple explanation.

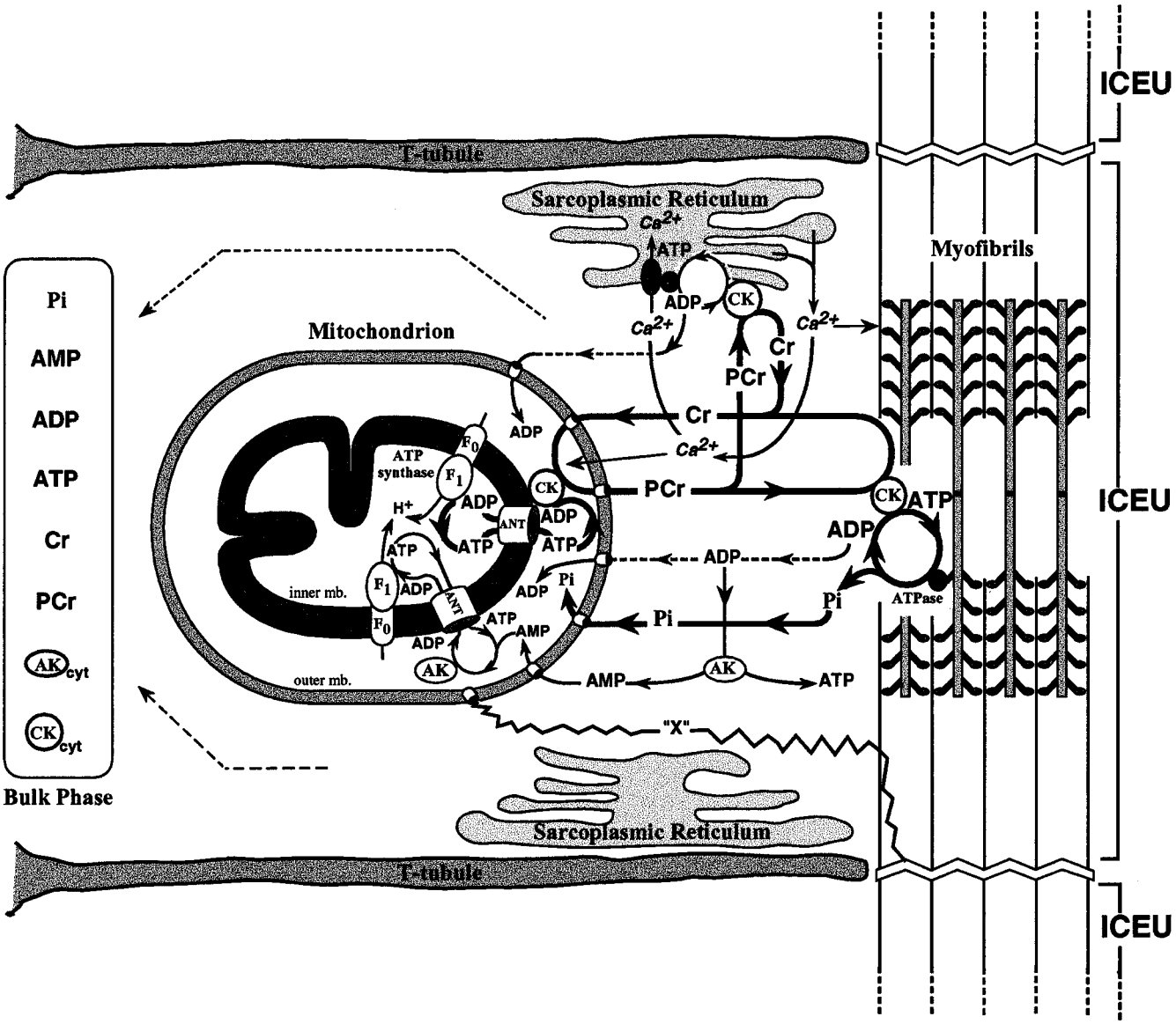
(1) Analysis of the diffusion of ADP between the bulk-water phase in the cell interior and the medium, by taking D equal to that in water, has shown that the rate of this exchange in water is much more rapid than the ADP-ATP turnover rate inside the fibres. This is in accordance with many previous calculations of 'diffusion distance, d', which corresponds to displacement of ADP molecules for the time of ADP turnover in mitochondria, τ. Using the Einstein–Smoluchowski equation for three-dimensional Brownian movement:

$$d^2 = 6D\tau \text{ [29]}$$

one can find that this distance d is 14 μm for $\tau = 100$ ms (Table 1) and clearly exceeds the size of the sarcomere and even the radius of the cardiomyocyte. Thus there are probably practically no ADP gradients between the bulk-water phases inside and outside the cells.

(2) The apparent K_m for exogenous ADP does not depend on the Mg-ATPase activity; it is the same without or with Ca^{2+} and Mg^{2+} (Table 2), in spite of the fact that, without Mg^{2+}, the ATPase activity is very low, whilst Ca^{2+} increases the Mg-ATPase activity (the activities differ at least 5-fold under these conditions).

(3) In rapid (glycolytic) muscles the myocyte diameter is three to five times larger than that found in red muscles. Thus one could expect significantly increased apparent K_m for exogenous ADP in white muscles. Experiments, however, show the contrary; in glycolytic muscles the apparent K_m for exogenous ADP is very low (7–10 μM) and does not differ from that for mitochondria *in vitro* [15,16]. When the red-fibre respiration was partially inhibited to precisely match mitochondrial activities (the rates of ADP consumption in the cells), the observed apparent K_m for exogenous ADP in red fibres still exceeded that in white fibres by



Scheme 1 Schematic presentation of functional ICEUs in the muscle cells

By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum are precisely fixed with respect to the structure of sarcomere of myofibrils between two Z-lines and correspondingly between two T-tubules. Ca^{2+} is released from the sarcoplasmic reticulum into the space of the ICEU in the vicinity of the mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. [5,6,38–40]. Adenine nucleotides within the ICEU do not equilibrate rapidly with adenine nucleotides in the bulk-water phase. The mitochondria, the sarcoplasmic reticulum and Mg-ATPase of myofibrils are interconnected by metabolic channelling of reaction intermediates and energy transfer within the ICEU by the creatine kinase (CK)-phosphocreatine (PCr) and myokinase systems. The protein factors (still unknown and marked as 'X'), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP [26]. This increases the microcompartmentation of adenine nucleotides within the ICEU and the importance of processes of vectorial ligand conduction [13], instead of free diffusion, as a basis of macro-osmotic processes. The ATP in the bulk-water phase may constitute a cellular metabolic reserve or serve some regulatory purposes. Adenine nucleotides within the ICEU and bulk-water phase may be connected by some more rapidly diffusing metabolites as creatine (Cr), -PCr. Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, P_i or PCr) and/or synchronized release of Ca^{2+} during the excitation-contraction coupling process. Abbreviation: AK, adenylate kinase.

an order of magnitude, despite of the smaller diameter of the former [15].

(4) Initially, it was proposed that the high apparent K_m for exogenous ADP might be the result of diffusion problems for ADP in myofibrils [37]. However, extraction of myosin by 800 mM KCl did not have any effect on the value of the apparent K_m for exogenous ADP (Figure 3; see also [27]). At the same time, it was discovered that disruption of the mitochondrial outer membrane by hypo-osmotic treatment of the permeabilized fibres decreases the apparent K_m for exogenous ADP to very low values, close to those for isolated mitochondria *in vitro* [27]. It was also found that the presence of creatine (due to continuous regeneration of the local ADP behind the mitochondrial outer membrane), in the intermembrane space of mitochondria in the coupled-mitochondrial-creatine-kinase reaction, decreases the apparent K_m for exogenous ADP severalfold [27,37].

(5) A significant decrease in the apparent K_m for exogenous ADP in regulation of mitochondrial respiration *in vivo* is achieved by short, rather selective, proteolytic treatment of skinned muscle cells and hepatocytes without any visible changes in cell geometry or enzyme activities, such as Mg-ATPase (Figures 6–9 and [16]).

These results show that the high apparent K_m values for exogenous ADP are tissue-specific and depend on the expression of some specific proteins, which are very sensitive to proteolytic digestion, in red muscle cells. Thus there are specific, probably localized, limitations of diffusion of ADP in these cells which depend upon the protein composition and supramolecular organization of the cell. It was originally proposed that some cytoplasmic proteins (then called factor 'x', unidentified proteins probably associated with cytoskeleton) may control the voltage-dependent-anion-channel (VDAC) permeability for ADP and ATP. These proteins may also take part in distribution of mitochondria in the cells by building up structurally and functionally organized pathways of feedback signalling between ATP-consuming (ADP-supplying) systems and mitochondria [12,13,26]. Due to such a structural supramolecular organization, the mitochondria become less easily accessible to exogenous ADP and endogenously generated ADP does not equilibrate easily with the ADP in the medium. While the nature of factor(s) 'x' is still unclear, the results of the present study show that there is clear organized functional relationship between the Mg-ATPases of the myofibrils and of the sarcoplasmic reticulum and mitochondria, with respect to metabolic channelling of ADP. This close structural organization contributes to the high apparent K_m for exogenous ADP in the regulation of respiration, whilst, at the same time, most of the endogenously generated ADP is not accessible to the exogenous PK and PEP if the mitochondrial oxidative phosphorylation is actively running. Since exogenous PK can easily occupy the intracellular bulk-water phase from where the LDH has been released during permeabilization, these results mean that the endogenously generated ADP is not immediately released into this bulk-water phase. Recent confocal-microscopy studies of Ca^{2+} release from the sarcoplasmic-reticulum mitochondria by using fluorescent Ca^{2+} probes and Ca^{2+}-sensitive photoprotein targeted to the outer phase of the mitochondrial inner membrane showed the focal release of Ca^{2+} into the vicinity of mitochondria [38–40]. Duchen [40] proposed that the sarcoplasmic reticulum and mitochondria 'may be intimately associated as a functional unit'. In the present study we show that not only Ca^{2+}, but also ADP, is focally released from the Mg-ATPases of both the reticulum and myofibrils into the vicinity of mitochondria. These functional units between Mg-ATPases and mitochondria may be called ICEUs as discussed above, since they seem to serve as a basic pattern of functional organization of the whole cell energetics. Scheme 1 illustrates this concept; it is supposed that due to their precisely fixed position in the cells (most probably by interaction with cytoskeletal elements), mitochondria, a sarcomere and adjacent sarcoplasmic reticulum (its two cisterns and a set of longitudinal tubules), all that exists between two Z-bands and two T-tubules form an effective functional unit, ICEU. In cardiac cells, the ICEUs may be revealed by imaging techniques as a very regular arrangement of mitochondria (compare Figure 1C and Scheme 1) [39,40]. It is proposed that nearly every sarcomere (or group of adjacent sarcomeres) has its own energy supply and regulatory systems. ADP, released focally into these units, does not equilibrate rapidly with the ADP in the medium, due to anisotropy of diffusion or channelling because of structural restrictions. We have shown directly that proteolytic degradation of the ICEU structure increases the access of exogenous ADP to mitochondria and ATP to Ca,Mg-ATPases (Figure 7, Table 3). There may also be significant restriction of ADP diffusion within these units at the level of the mitochondrial outer membrane (control of VDAC and/or in myofibrils) since our experiments (Figures 5 and 6) show directly that both ADP diffusion and energy transfer are facilitated by activation of the energy-transfer network, the creatine kinase system. This limitation of ADP diffusion at the mitochondrial membrane increases the compartmentation of adenine nucleotides within the ICEU. Thus all that has been told about the organization and function of the creatine kinase system [26,41,42] seems to be relevant to the organization of the ICEU, as is shown schematically in Scheme 1.

In conclusion, it is proposed that the supramolecular organization of the ICEU described above increases the efficiency of interaction between mitochondria, myofibrils, the sarcoplasmic reticulum and probably other energy-consuming systems both by mechanisms of channelling and compartmentation of ADP within the ICEU in the vicinity of mitochondria (there is no need for ADP to diffuse in the whole cell). Furthermore, this ADP enters the creatine kinase and adenylate kinase networks of energy transfer to stimulate mitochondrial energy production. Participation of these networks in regulation is necessary to avoid rapid accumulation of ADP within the ICEUs and thus to avoid product inhibition of the ADP-producing systems, contraction and ion transport, as discussed previously [26,41,42]. One may propose that, while there is restricted diffusion of ADP between neighbouring ICEUs because of their supramolecular organization, smaller metabolites, such as P_i, creatine and also phosphocreatine, could diffuse more freely and participate in synchronizing the functional activities of different ICEUs in the cell. Interestingly, most of the cellular ATP seems to be localized in the bulk phase of the cell water, since Neely et al. [11] have shown that 70% of cellular ATP can be easily removed without any effect on the cardiac contractile function.

This specific structure, ICEU, falls apart after short, probably selective, proteolysis of some structural proteins (Figure 9), while the activities of enzyme are not changed by this treatment (Figure 7). This explains the empirical observation that treatment of muscle homogenates with proteases is a necessary step in the isolation of intact mitochondria. However, the existence of ICEUs may not be limited to the striated muscle cells. Mannella et al. [43] showed by electron microscopic tomography that in hepatocytes mitochondria form clusters with stacks of endoplasmic reticulum. In addition, an ordered arrangement of mitochondria in hepatocytes due to clusters of mitochondrial–rough endoplasmic reticulum associations has also been shown by scanning electron microscopy [44]. Multiple connections between mitochondria and cytoskeletal elements have been described in muscle and other types of cells, giving rise to the concept of a mitochondrial reticulum [45–47]. Thus mitochondrial distribution in the cell due to these interactions seems to be directly related to the control of their function. However, identification of the proteins responsible for both these important phenomena requires further study.

The clear differences between the metabolites and enzymes in the bulk phase of intracellular water, and inside the organized metabolic systems and multienzyme complexes, observed in the present study are in good accord with theoretical concepts of cell architecture and metabolic channelling developed by Ovadi [48] and Srere [49]. This concept of eukaryotic cell organization considers that virtually all the cell architecture is interconnected (by the 'microtrabecular lattice' containing cytoskeletal elements) with intervening aqueous phase(s).

This type of organization of whole metabolic systems into functional units where the intermediates are exchanged by metabolic channelling is not only a characteristic for the energy

metabolism of muscle cells. Deutscher et al. [50] have described in a series of important works the supramolecular organization and substrate channelling in the mammalian translation system during protein synthesis *in vivo*. By using permeabilized cells, it was shown that a pool of ^{14}C-labelled aminoacyl-tRNAs synthesized endogenously did not mix with their exogenously supplied counterparts, despite the fact that the latter are distributed throughout the cell. These data showed that there is a channelled tRNA cycle during protein synthesis in mammalian cells. It was also shown that such an organization and channelling in the protein synthesis machinery is related to the actin microfilament network [50]. Similar data were reported previously by Cheung et al. [51].

The concept of the ICEU developed in the present paper helps to explain two very important phenomena whose mechanisms are still unclear. First is the mechanism of cardiac-contraction failure under conditions of inhibition of oxidative phosphorylation, such as ischaemia or hypoxia, when the cellular content of ATP is still high. Multiple theories, such as increased P_i or altered Ca^{2+}-sensitivity of myofibrils etc., have failed to explain why in the presence of 70–80 % of cellular ATP contraction stops and soon afterwards contracture (a spontaneous increase in the end-diastolic pressure) develops, while still in the presence of some ATP. The concept of the ICEU gives an explanation in agreement with the original hypothesis of Gudbjarnason et al. [10] that this is a pool of ATP inside the ICEU connected to the phosphocreatine content that is rapidly used up under these conditions, resulting in the accumulation of ADP inside the ICEU, the inhibition of cross-bridge detachment and thus inhibition of contraction despite the presence of ATP in the bulk-water phase of the cell.

The second phenomenon is the recently described metabolic heterogeneity of mitochondria in cardiomyocytes under conditions of substrate deprivation, recently seen by confocal microscopy [52]. Romashko et al. [52] showed that mitochondria in different parts of the cell may be oxidized to different extents, and the redox potential of the respiratory chain can change spontaneously, by metabolic oscillations and waves. This may correspond to differences in the behaviour of different ICEUs when synchronization of their function is disturbed. The cellular mechanism of the synchronization of the ICEUs remains unresolved.

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