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Intracellular compartmentation of cardiac fibres from rainbow trout and Atlantic cod—a general design of heart cells

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Abstract

In mammalian cardiomyocytes, mitochondria and adjacent ATPases with participation of creatine kinase (CK) constitute functional compartments with an exchange of ADP and ATP delimited from cytosolic bulk solution. The question arises if this extends to ectothermic vertebrates: their low body temperature and thinner cardiomyocytes with a lower density of membrane structures may reduce the need and structural basis for compartmentation. In saponin-skinned cardiac fibres from rainbow trout and Atlantic cod, we investigated mitochondrial respiration induced by endogenous ADP generated by ATPases and its competition for this ADP with pyruvate kinase (PK) in excess. At low Ca^{2+} activity (pCa = 7.0), PK lowered ATP-induced respiration by 40% in trout and 26% in cod. At high Ca^{2+} activity (pCa = 5.41), PK had no effect. Additionally, ADP release from the fibres was almost zero but increased drastically upon inhibition of respiration with 1 mM Na-azide. This suggests that fibres are compartmented. PK abolished creatine-stimulated respiration in trout suggesting a less tight coupling of CK to respiration than in mammals. In conclusion, intracellular compartmentation seems to be a general feature of vertebrate cardiomyocytes, whereas the role of CK is unclear, but it seems to be less important for energy transport in species with lower metabolism.

Keywords: Ectothermic vertebrate; Rainbow trout; Atlantic cod; Cardiomyocyte; Mitochondrial respiration; Intracellular compartmentation

1. Introduction

In cardiomyocytes, in which the main energy source is mitochondrial oxidative phosphorylation, extended periods of high workload require a very fast and efficient feedback of ADP and ATP between mitochondria and ATPases. It has been proposed that in mammalian cardiomyocytes, the feedback is enhanced by compartmentation into "intracellular energetic units" (ICEUs) [1] within which ADP and ATP are channelled between mitochondria and adjacent myofibrils and sarcoplasmic reticulum (SR) [1–3]. Inside the ICEUs, functional coupling of creatine kinase (CK) to on the one hand ATPases of the myofilaments and the SR membrane [4,5] and on the other hand the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane [6] may further enhance the feedback.

We speculated whether cardiomyocytes from ectothermic vertebrates exhibit a similar compartmentation. Their body temperature is close to the environmental temperature and typically much lower than in mammals. A lowered temperature should enhance the diffusion flux ($Q_{10} \sim 1.3$) [7] relative to metabolic rate ($Q_{10} \sim 2$) [8]. Furthermore, even at the same body temperature, the energy metabolism of an ectothermic vertebrate is much lower than that of a mammal of similar size [9]. Therefore, diffusion flux relative to metabolic rate should be much higher in ectothermic vertebrates and tentatively the need for compartmentation much lower. In addition, the cardiomyocytes of ectothermic vertebrates are structurally different from those of mammals [10,11]. Thus, fish cardiomyocytes generally have much smaller diameters (~5 μm for rainbow trout) than those from rats (~20 μm) [12,13]. Furthermore, they lack T-tubules [12] and have a much lower density of SR [14].

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These factors may reduce the structural basis for compartmentation, as intracellular diffusion is likely to be restricted mainly by membranes [15].

Despite the above arguments against compartmentation, our previous study on skinned cardiac fibres from rainbow trout and Atlantic cod at 10 °C showed that the mitochondrial apparent ADP affinity is much lower than that of isolated mitochondria. This supports the existence of intracellular compartments delimited from the bulk cytoplasm [16]. Furthermore, in trout but not in cod cardiomyocytes, the low apparent ADP affinity was increased in the presence of creatine, suggesting a functional coupling of CK to mitochondrial respiration [16]. The lack of a creatine effect in skinned cod fibres is interesting, because when comparing different types of mammalian muscles, a low apparent mitochondrial ADP affinity in skinned fibres is associated with a functional coupling of CK to respiration [17].

The aim of the present study was to further assess the delimitation of intracellular ADP transport from the cytoplasmic bulk solution in skinned cardiac fibres from rainbow trout and Atlantic cod. This was done in terms of the competition for ADP generated by endogenous ATPases between mitochondrial respiration and PK added in excess to the surrounding medium. ADP was generated by endogenous ATPases following addition of ATP to the respiratory medium. When a steady-state respiration rate had been achieved, phosphoenolpyruvate (PEP) was added to the medium to activate the reaction of PK present in the medium, and the effect of this on respiration was recorded. Without intracellular compartmentalization, ADP produced by ATPases should be consumed by PK almost completely before reaching the mitochondria, because the ADP consumption capacity of PK exceeded that of the mitochondria by at least 100 times. The experiments were carried out at low (pCa = 7.0) and high Ca^{2+} activity (pCa = 5.41) to assess the importance of Ca^{2+}-activated ATPases and in the absence and presence of creatine to assess the role of CK.

2. Materials and methods

Rainbow trout (*Oncorhynchus mykiss*) (262 ± 12 g) were obtained from a local fish farm (Pinds Mølle, Denmark). They were kept in freshwater tanks at 10–12 °C and fed regularly with commercial trout pellets. Atlantic cod (*Gadus morhua*) (397 ± 24 g) were caught in the Kattegat at least 3 weeks before the experiments. They were kept in salt-water tanks at 10–12 °C and fed regularly with small crabs.

The experimental animal was killed by a blow to the head and decapitation. The heart was quickly excised and transferred to an ice-cold physiological solution. The physiological solution for trout was composed of (mM) 125 NaCl, 2.5 KCl, 0.95 $MgSO_4$, 1.5 $CaCl_2$ and 5 HEPES and brought to pH 7.6 at 10 °C with imidazole. The same solution was used for cod with the exception that NaCl was 150 mM. The ventricle was isolated and washed free of blood. A small piece of the apex was cut off and frozen in liquid nitrogen for later determination of enzyme activities in whole-tissue homogenate. The rest of the ventricle was used immediately in the skinned fibre experiments.

2.1. Skinning of muscle fibres

The ventricle was isolated and transferred to a small dish containing ice-cold skinning solution (S-solution) composed of (mM) 10 EGTA–Ca–EGTA buffer, 1.38 $MgCl_2$, 20 imidazole, 20 taurine, 0.5 dithiothreitol (DTT), 50 KOH, 50 mM methane sulfonic acid, 5.7 mM Na_2ATP and 15 mM PCr. The pH was 7.3 at 10 °C. The free Ca^{2+} of this solution was 0.1 μM (pCa = 7) as calculated by a computer program on the basis of the binding constants for Ca^{2+} and Mg^{2+} to a series of substances including EGTA and ATP and the dependence of these constants on pH, temperature and ionic strength [18].

In small muscle pieces, fibres were gently separated with fine forceps to obtain a network of cells only loosely connected at a few sites. Trout fibres were incubated for one hour with intense shaking (600 rpm in a Sarstedt CM-9 Combi-Mischer) in S-solution containing 100 μg ml^{-1} saponin as in a previous experiment [19]. Cod fibres, which are more fragile, were incubated for 30 min with less intense shaking (200 rpm in a Sarstedt CM-9 Combi-Mischer) in S-solution containing 75 μg ml^{-1} saponin. By acting on cholesterol, saponin selectively perforates the sarcolemma, leaving the membranes of the mitochondria and sarcoplasmic reticulum and their contacts with the cytoskeleton intact [20]. After skinning, the fibre bundles were washed in a saponin-free S-solution for 10 min with intense shaking and left in the solution for at most 3 h until use in the respiration experiments. All these preparative procedures were carried out at 0 to 4 °C.

2.2. Mitochondrial respiration rate

Skinned fibres from all individuals were used for respiration experiments. Furthermore, from some individuals, a fraction of the skinned fibres was frozen in liquid nitrogen for later determination of enzyme activities. Immediately before being transferred to the respiratory chamber or frozen, the fibres were washed twice for 5 min in respiration solution (R-solution) in order to remove adenosine phosphates, creatine and phosphocreatine. The R-solution was composed of (mM) 10 EGTA–CaEGTA buffer, 1.38 $MgCl_2$, 20 imidazole, 20 taurine, 0.5 dithiothreitol (DTT), 90 KOH, 10 NaOH, 10 mM methanesulfonic acid, 3 K_2HPO_4, and 5 glutamic acid and 2 malic acid as respiratory substrates. The pH of the R-solution was 7.3 at 10 °C. The Ca–EGTA buffer provided pCa = 7.0 except for experiments in which free Ca^{2+} was elevated to pCa = 5.41 by the addition of $CaCO_3$ and subsequent readjustment of pH to 7.3 with imidazole. The low Ca^{2+} activity, pCa = 7.0, resembles that in the resting cardiac cell [21]. The high Ca^{2+} activity, pCa = 5.41, which has been recorded in frog myocardium and is within a range likely to be attained in activated trout cardiac cells [21], elicits the maximal activity at 10 °C of cardiac myosin-ATPase in homogenate from rainbow trout [22].

Mitochondrial respiration of the fibres from each ventricle was recorded in two set-ups run in parallel. In each set-up, fibres were incubated in 3 ml R-solution under continuous stirring. The 3 ml R-solution filled a thermostated (Hetofrig, Denmark) glass chamber with a 30-mm high glass stopper on top. This stopper was pierced by a tube with a diameter of 1.2 mm through which additions to the solution could be made with a syringe (Hamilton Microliter 801). The oxygen tension of the solution was recorded with an oxygen electrode (Radiometer E5046) situated horizontally in a tightly fitting glass tube with its tip just inside the chamber. Oxygen tension values were sampled and stored every 2 s by a computer program. All experiments were conducted at 10 °C.

The respiratory experiments consisted of two main groups, involving different specimens of trout and cod. The first group aimed at the kinetics of mitochondrial respiration stimulated with exogenous ADP and ATP at low and high Ca^{2+} activity, and the second at the effect of an exogenous ADP-trap (PK and PEP) on mitochondrial respiration stimulated with endogenous ADP generated by ATPases upon addition of 1 mM ATP.

2.3. Kinetics of mitochondrial respiration stimulated with exogenous ADP and ATP at low and high Ca^{2+} activity

Four skinned fibre preparations were obtained from each experimental animal to record ADP and ATP dependency of respiration at low and high Ca^{2+} activity. The ADP or ATP concentration in the chamber was increased in successive steps, with recordings of the stabilized respiratory rate at each step. According to experiments on rat heart, the respiratory rate at a given ADP concentration obtained by successive additions of ADP does not differ from that obtained by addition of ADP in one step [2]. Each experiment was terminated by collecting the fibre bundles contained in the respiratory chamber for dry weight determination. The fibre bundles were placed on a small piece of aluminium paper, dried at 70 °C for 1 h, and weighed.

Before addition of ADP, the state IV respiration, i.e., the basal respiration, V_0, was recorded. This respiration is not associated with phosphorylation of ADP and is probably due to a proton leak across the inner mitochondrial

membrane [23]. The ADP- or ATP-stimulated respiration rate, V_{ADP} or V_{ATP}, was obtained after subtraction of V_0 from the respiratory rate recorded in the presence of ADP or ATP.

The respiratory rates are expressed as nmol O_2 min^{-1} mg dry $weight^{-1}$. V_{ADP} and V_{ATP} were plotted as a function of the concentrations of ADP added to the respiratory medium. Apparent $K_{M\ ADP}$ and $K_{M\ ATP}$, respectively, and maximal respiration rates elicited by ADP and ATP, $V_{ADP\ max}$ and $V_{ATP\ max}$, were determined by fitting the data to the Michaelis-Menten equation written according to Hanes [24]. Acceptor-control ratios were calculated: ACR_{ADP} as $(V_{ADP\ max} + V_0)/V_0$ and ACR_{ATP} as $(V_{ATP\ max} + V_0)/V_0$.

2.4. Effects of an exogenous ADP-trapping system on mitochondrial respiration stimulated with endogenous ADP generated by non-specific ATPases, Ca^{2+}-activated ATPases and CK

Six skinned fibre preparations were obtained from each experimental animal in order to run three experiments each with two preparations, one at low and one at high Ca^{2+} activity. Fig. 1 depicts representative traces of the three experiments with rainbow trout at low Ca^{2+} activity. In the first experiment, mitochondrial respiration was stimulated by the addition of 1 mM ATP to the respiratory solution, i.e., mitochondrial respiration rate was recorded first in the absence and then in the presence of 1 mM ATP (Fig. 1). In the second experiment, being an extension of the first, the respiration solution additionally contained 10 U/ml PK, and after recording of steady-state respiration rate in the presence of 1 mM ATP, 5 mM PEP was added to the solution to activate the PK reaction and its competition with the mitochondria for endogenous ADP generated by cellular ATPases (Fig. 1). The third experiment was identical to the second with the exception that the respiration solution additionally contained 20 mM creatine to assess the importance of CK (Fig. 1).

The experiments were concluded by collecting the fibres for dry weight determination. Furthermore, in the first experiment without PK, 400 µl 70% perchloric acid was added to the respiratory medium to stop enzymatic reactions. This acidified chamber solution was collected for determination of ADP (see below).

The ATP-stimulated respiration rate, V_{ATP}, was obtained after subtraction of V_0 from the respiratory rate (nmol O_2 min^{-1}·mg dry $weight^{-1}$).

The activity of PK (2 µmol min^{-1} ml^{-1} at 10 °C) and PEP (5 mM) added to the respiratory medium should yield a potential ADP consumption rate of PK which is several-fold higher than that of the mitochondria. Indeed, assuming a P/O ratio of 3, $V_{1\ mM\ ATP}$ corresponds to an ADP-consumption rate ranging from 6 to 15 nmol min^{-1} mg dw^{-1}. As the mass of the fibres in the 3 ml respiration chamber never exceeded 3 mg dw, the potential ADP consumption rate of PK was at least hundred-fold higher than that of the mitochondria.

2.5. Time dependency of ADP release

The mitochondrial capacity to consume endogenous ADP was further assessed in an experiment in which the release of ADP from the fibre preparation into the surrounding medium was recorded as a function of time. Fibre bundles were incubated in R-solution with 1 mM ATP contained in a cuvette placed in a spectrophotometer. Additionally, the solution contained pyruvate kinase, lactate dehydrogenase, 0.5 mM phosphoenolpyruvate and 0.7 mM NADH so that the formation of ADP could be continuously recorded spectrophotometrically at 340 nm [25]. When steady state had been reached, 1 mM Na-azide was added to block mitochondrial respiration [2]. These measurements were conducted at both low and high Ca^{2+} activity.

2.6. Determination of ADP concentration in the respiratory solution

The acidified respiration solution was centrifuged at 17,000×g for 10 min. The supernatants were neutralised by the addition of 3 ml 2 M $KHCO_3$. The new supernatant obtained after centrifugation for 10 min was stored at −80 °C for determination of ADP. The ADP concentration was measured spectrophotometrically at 340 nm in an assay coupled to pyruvate kinase and lactate dehydrogenase [25].

2.7. Enzyme activities

Frozen tissue samples were weighed and placed in small glass homogenisers. Homogenisation buffer was added to obtain a tissue concentration of 50 mg/ml. The homogenisation buffer was composed of (mM) 5 HEPES, 1 EGTA, 1 DTT and 0.10% Triton X-100. The pH was adjusted with KOH to 8.7 at 10 °C. The tissue was manually homogenised, and the homogenate was incubated for 1 h with stirring every 15 min.

Citrate synthase activity was recorded in an assay in which its reaction was coupled to the production of thionitrobenzoic acid (TNB). The rate of TNB-production was recorded spectrophotometrically at 412 nm in a reaction buffer composed of (mM) 100 Tris pH 8, 0.1 DTNB, 0.3 acetyl CoA and 0.5 oxaloactic acid.

Pyruvate kinase activity was recorded in an assay in which the formation of ATP was coupled to the oxidation of NADH to NAD by the reaction of lactate dehydrogenase. The rate of NADH oxidation was recorded spectrophotometrically at 340 nm. The reaction buffer was composed of (mM) 50 TEA, 75 KCl, 8 MgSO4, 1 PEP, 1 ADP, 0.3 NADH and 3 U/ml lactate dehydrogenase, and its pH was adjusted with KOH to 7.5 at 25 °C.

Lactate dehydrogenase activity was recorded by following the rate of NADH oxidation spectrophotometrically at 340 nm. The reaction buffer contained (mM) 50 TEA, 75 KCl, 8 MgSO4, 0.067 NADH and 0.35 Na^+-pyruvate, and pH was adjusted with KOH to 7.5 at 25 °C. After the initial recording at 0.35 mM Na^+-pyruvate, the pyruvate concentration was increased to 10 mM by addition from a

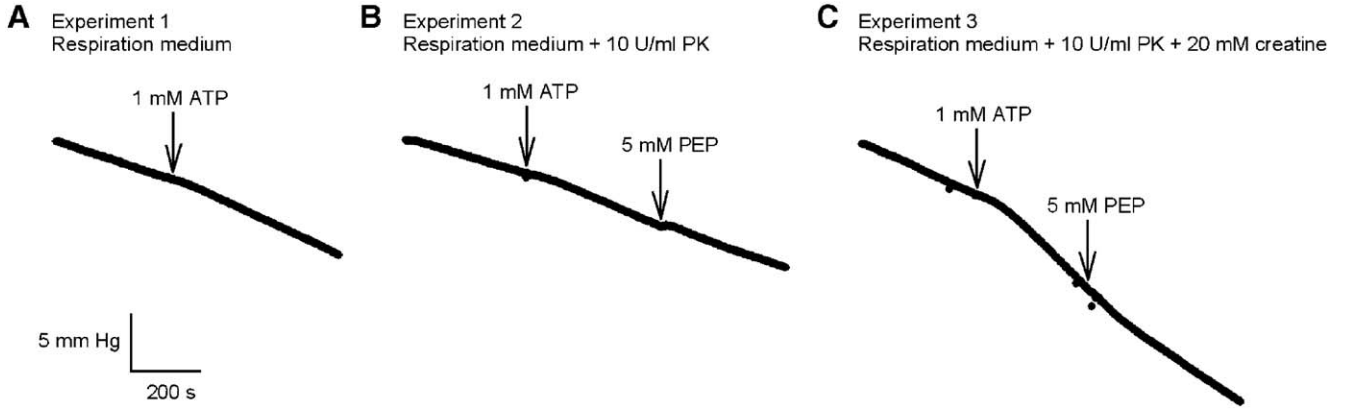

Fig. 1. The three types of experiments used to assess ATP-stimulated respiration and the effect of activating PK to compete with mitochondria for endogenous ADP generated by ATPases. Figure shows representative traces from rainbow trout at low Ca^{2+} activity. (A) In the first experiment, mitochondrial respiration rate was stimulated by the addition of 1 mM ATP to the respiratory medium. (B) In the second experiment, the respiratory medium also contained 10 U/ml PK, which following addition of 5 mM PEP should compete with the mitochondria for consumption of endogenous ADP. (C) The third experiment was the same as the second, but the respiratory medium additionally contained 20 mM creatine to assess the importance of CK.

Table 1
ADP and ATP dependency of mitochondrial respiration in skinned fibres recorded at low and high Ca^{2+} activity

	Ca^{2+} activity	V_0 nmol O_2 min^{-1} mg^{-1}	$V_{ADP\ max}$ nmol O_2 min^{-1} mg^{-1}	ACR_{ADP}	$K_{M\ ADP}$ μM	$V_{ATP\ max}$ nmol O_2 min^{-1} mg^{-1}	ACR_{ATP}	$K_{M\ ATP}$ μM
Trout (4)	Low	1.85±0.08	11.74±0.83	7.63±0.67	733±31	1.66±0.12$^{\#\#}$	1.88±0.09$^{\#\#}$	186±55$^{\#\#\#}$
	High	2.60±0.16	6.20±0.78*	3.68±0.27*	327±68**	3.21±0.33*,$^{\#}$	2.11±0.10$^{\#\#}$	197±27
Cod (5)	Low	2.19±0.16	9.32±1.70	5.82±0.82	680±80	3.65±0.54	2.48±0.09$^{\#}$	301±49$^{\#}$
	High	2.52±0.17	4.47±0.27*	2.94±0.07*	128±26**	2.91±0.28$^{\#}$	2.09±0.09**,$^{\#}$	126±36*

*$P<0.05$ and **$P<0.01$ significant effect of high Ca^{2+} activity.
$^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ significantly different from the value obtained when stimulating with ADP.
Oxygen consumption was recorded polarographically in either low (pCa=7) or high (pCa=5.41) Ca^{2+} after addition of increasing concentrations of either ADP or ATP. V_0 is the respiratory rate in the absence of ADP or ATP. $V_{ADP\ max}$ and $V_{ATP\ max}$ are the maximal rates obtained following increases in ADP and ATP, respectively, and $K_{m\ ADP}$ and $K_{m\ ATP}$ are the concentrations of ADP and ATP eliciting half of $V_{ADP\ max}$ and $V_{ATP\ max}$, respectively. These parameters were determined by plotting the data according to Hanes. $ACR_{ADP} = \frac{V_0 + V_{maxADP}}{V_0}$ and $ACR_{ATP} = \frac{V_0 + V_{maxATP}}{V_0}$. Results are mean±SEM. Number of animals within brackets.

stock solution of 0.5 M, and the pyruvate inhibition ratio was calculated as the ratio between the activities at 0.35 and 10 mM pyruvate [26]. According to the model based on studies of mammals and birds, lactate dehydrogenase exists as a tetramer composed of two types of monomers; H-type and M-type. The H-type has a higher affinity for pyruvate than the M-type but is allosterically inhibited at high concentrations of pyruvate [27]. Therefore, a pyruvate inhibition ratio above or below 1 suggests that the H-type or the M-type, respectively, is the prevalent isoform [26].

Adenylate kinase (AK) and creatine kinase (CK) activity were recorded in an assay in which the formation of ATP was coupled to the reduction of NADP to NADPH by the reactions of hexokinase and glucose-6-phosphate-dehydrogenase. The rate of NADPH-formation was recorded spectrophotometrically at 340 nm. The reaction buffer was composed of (mM) 20 HEPES, 5 MgAcetate, 0.5 DTT, 20 glucose, 1.2 ADP, 0.6 NADP, 5 U/ml hexokinase and glucose-6-phosphate-dehydrogenase. Its pH was adjusted with NaOH to 7.5 at 25 °C. First, the activity of adenylate kinase was recorded after addition of homogenate. Then 9.8 mM PCr was added to the cuvette, and the activity of both adenylate kinase and creatine kinase was recorded. Creatine kinase activity was calculated by subtraction of the adenylate kinase activity.

The activities were obtained as μmol min^{-1} g ww^{-1}. Recordings performed on skinned fibres were used to estimate the remaining fraction of CK by relating to the activity of citrate synthase.

2.8. Data handling and statistics

Results are presented as mean ± S.E.M. The results were analysed using Student's t test. $P < 0.05$ was considered significant.

3. Results

3.1. Kinetics of mitochondrial respiration stimulated with exogenous ADP and ATP at low and high Ca^{2+} activity

ADP dependency of mitochondrial respiration recorded at low and high Ca^{2+} activity yielded similar results for trout and cod (Table 1). An increase in Ca^{2+} activity decreased $V_{ADP\ max}$ and ACR. Additionally, the apparent $K_{M\ ADP}$ determined with respect to the ADP concentration added to the respiratory medium was decreased. This effect appeared to be larger for cod than for trout inasmuch as its $K_{M\ ADP}$ at high Ca^{2+} activity was lower than that recorded for trout.

The kinetics of mitochondrial respiration stimulated with exogenous ATP was very different from those obtained when stimulating with exogenous ADP (Table 1). $V_{ATP\ max}$ was lower than $V_{ADP\ max}$ (however, for cod at low Ca^{2+} activity, this was only close to significance, $P = 0.0527$). As a result, ACR_{ATP} was lower than ACR_{ADP}. As previously suggested, the ATPase activity might be low relative to the mitochondrial ATP synthase activity whereby $V_{ATP\ max}$ and ACR_{ATP} will be low [19]. An increase in Ca^{2+} activity increased $V_{ATP\ max}$ in trout but not in cod. The apparent $K_{M\ ATP}$ was much lower than $K_{M\ ADP}$ at low Ca^{2+} activity. However, at high Ca^{2+} activity, $K_{M\ ATP}$ and $K_{M\ ADP}$ were similar.

3.2. Effects of an exogenous ADP-trapping system on mitochondrial respiration stimulated with endogenous ADP generated by non-specific ATPases, Ca^{2+}-activated ATPases and CK

The effect of exogenous PK and PEP trapping endogenous ADP was investigated in three types of experiments, which are shown in Fig. 1. The three experiments were run at both low and high Ca^{2+} activity.

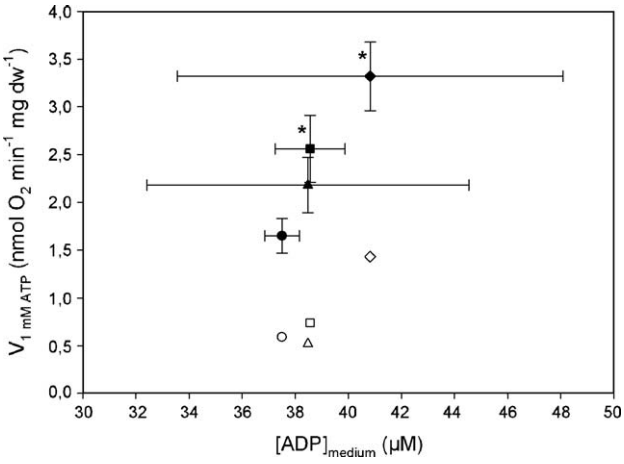

Fig. 2. Mitochondrial respiration stimulated with 1 mM ATP, $V_{1\ mM\ ATP}$, plotted as a function of the associated endogenous ADP concentration recovered in the respiratory medium, $[ADP]_{medium}$ (results from experiment 1 in Fig. 1). Recordings at low and high Ca^{2+} activity are shown with filled symbols with error bars. Open symbols show the expected respiration rate if respiration had been stimulated with the same concentration of exogenous ADP, V_{ADP}. This was calculated on basis of the Michaelis-Menten equation and the results from Table 1. Symbols: ● and ○, Rainbow trout at low Ca^{2+}; ■ and □, Rainbow trout at high Ca^{2+}; ▲ and △, Atlantic cod at low Ca^{2+}; ◆ and ◇, Atlantic cod at high Ca^{2+}. Results are mean±S.E.M. from five rainbow trout and six Atlantic cod. In all cases was $V_{1\ mM\ ATP}$ significantly higher than the calculated V_{ADP} ($P < 0.001$). At high Ca^{2+} activity, $V_{1\ mM\ ATP}$ relative to [ADP] was significantly higher than at low Ca^{2+} activity (*$P < 0.05$).

The first experiment assessed the effect of Ca^{2+} on $V_{1\ mM\ ATP}$ (respiration rate stimulated with 1 mM ATP) in relation to the concentration of endogenous ADP released into the surrounding respiratory medium, [ADP_{medium}]. Fig. 2 shows that $V_{1\ mM\ ATP}$ was increased by Ca^{2+} without any significant difference in [ADP_{medium}]. For comparison, we calculated for [ADP_{medium}] what the respiration rate would have been, if it had been stimulated with the same concentration of exogenous ADP added to the medium. This expected respiration rate, V_{ADP}, was calculated from the average [ADP_{medium}] using the Michaelis-Menten equation and the results from Table 1. V_{ADP} is included in Fig. 2 and is significantly lower than $V_{1\ mM\ ATP}$ in both trout and cod at both low and high Ca^{2+} activity. Thus, the mitochondria in skinned fibres have a higher apparent affinity for endogenous ADP generated by ATPases than for exogenous ADP added to the medium. Both these affinities are increased at high Ca^{2+} activity.

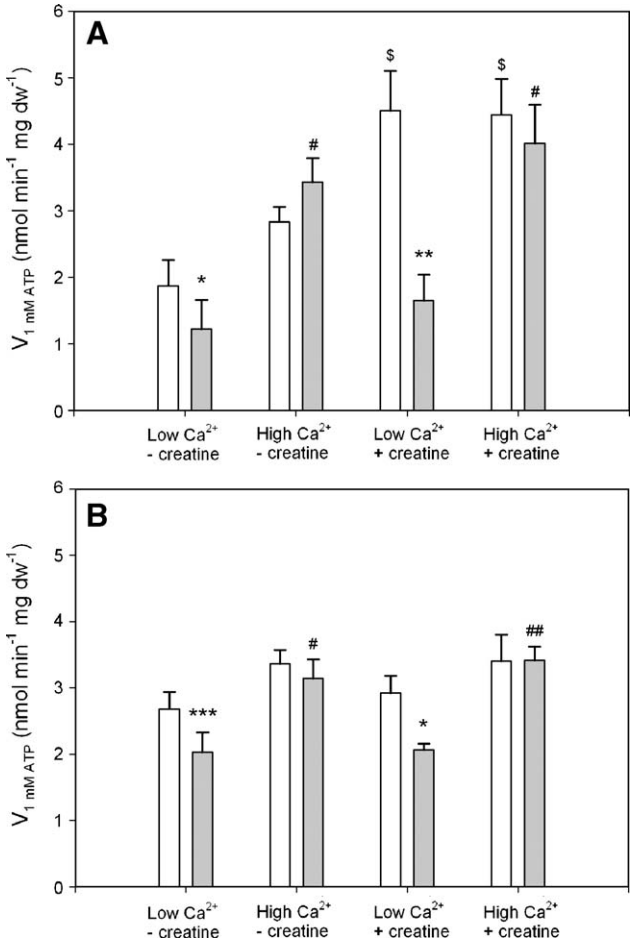

Fig. 3. The effect on respiration rate of activating PK to compete with mitochondria for consumption of endogenous ADP generated by ATPases. In skinned fibres from rainbow trout (A) and Atlantic cod (B), mitochondrial respiration was stimulated with 1 mM ATP at low and high Ca^{2+} activity and in the absence (results from experiment 2 in Fig. 1) and presence (results from experiment 3 in Fig. 1) of 20 mM creatine. The respiratory medium contained 10 U/ml PK from the beginning of the experiments. White bars and grey bars represent recordings before and after addition of 5 mM PEP to the medium, respectively. Results are mean±S.E.M. from five rainbow trout and six Atlantic cod. *$P<0.05$; **$P<0.01$ and ***$P<0.001$ significant effect of PK and PEP. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ significant effect of high Ca^{2+} activity. $^{\$}P<0.05$ significant effect of creatine.

Table 2
Basal respiration rate, V_0, from the three experiments in Fig. 1 recorded at low and high Ca^{2+} activity before addition of 1 mM ATP to the respiratory medium

		Experiment 1	Experiment 2	Experiment 3
Trout (5)	Low Ca^{2+}	1.63±0.26	1.72±0.33	2.21±0.24
	High Ca^{2+}	2.17±0.15	2.82±0.22*	2.85±0.26
Cod (6)	Low Ca^{2+}	2.06±0.14	2.23±0.15	2.89±0.10$^{\#}$
	High Ca^{2+}	3.12±0.24*	3.08±0.19*	2.71±0.26

*$P<0.05$, significant effect of high Ca^{2+} activity.
$^{\#}P<0.05$, significant effect of creatine.
V_0, i.e., the oxygen consumption rate before addition of ATP, was recorded in either low (pCa=7) or high (pCa=5.41) Ca^{2+}. In experiments 1 and 2 the respiratory medium was the same during V_0 recording, whereas in experiment 3, it contained in addition 20 mM creatine. See also text to Fig. 1. Results are mean±SEM. Number of animals within brackets.

The second experiment assessed how respiration rate is affected by initiating the reaction of PK competing with the mitochondria for endogenous ADP. At low Ca^{2+} activity, addition of PEP to the medium decreased respiration rate by 40±13% in trout (Fig. 3A) and 26±5% in cod (Fig. 3B), respectively. At high Ca^{2+} activity, the addition of PEP had no effect on respiration rate (Fig. 3A and B).

The third experiment assessed the importance of CK. Creatine affected respiration rate in trout only. At low Ca^{2+} activity, $V_{1\ mM\ ATP}$ was 184±63% higher in the presence than in the absence of creatine. At high Ca^{2+} activity, it was 58±17% higher (Fig. 3A). Thus, $V_{1\ mM\ ATP}$ was similar at high and low Ca^{2+} activity in the presence of creatine. Importantly, addition of PEP removed the effect of creatine (Fig. 3A). In cod, creatine did not influence $V_{1\ mM\ ATP}$ or the effect of activating the PK reaction (Fig. 3B).

3.3. Basal respiration rates

The basal respiration rate, V_0, was recorded before addition of ADP or ATP to the respiratory medium. It tended to be higher at the high relative to the low Ca^{2+} activity in the absence of creatine (Tables 1 and 2). This may be due to a direct effect of Ca^{2+} on mitochondria [28,29] or to the possibility that adenosine phosphates were not completely washed out of the fibres. However, this is not likely to influence the interpretation of the data as the ACR of fibres stimulated with exogenous ADP under control conditions (at low Ca^{2+} activity) was almost 6 or more (Table 1). In the presence of creatine, V_0 was similar at high and low Ca^{2+} activity in both trout and cod (Table 2, experiment 3).

3.4. Time dependency of ADP release

The channelling of ADP from ATPases to mitochondria was also examined by recording the rate of ADP release from skinned fibres stimulated with 1 mM ATP. Fig. 4A and C show representative recordings of the spectrophotometric NADH absorbance at 340 nm as a function of time from trout and cod,

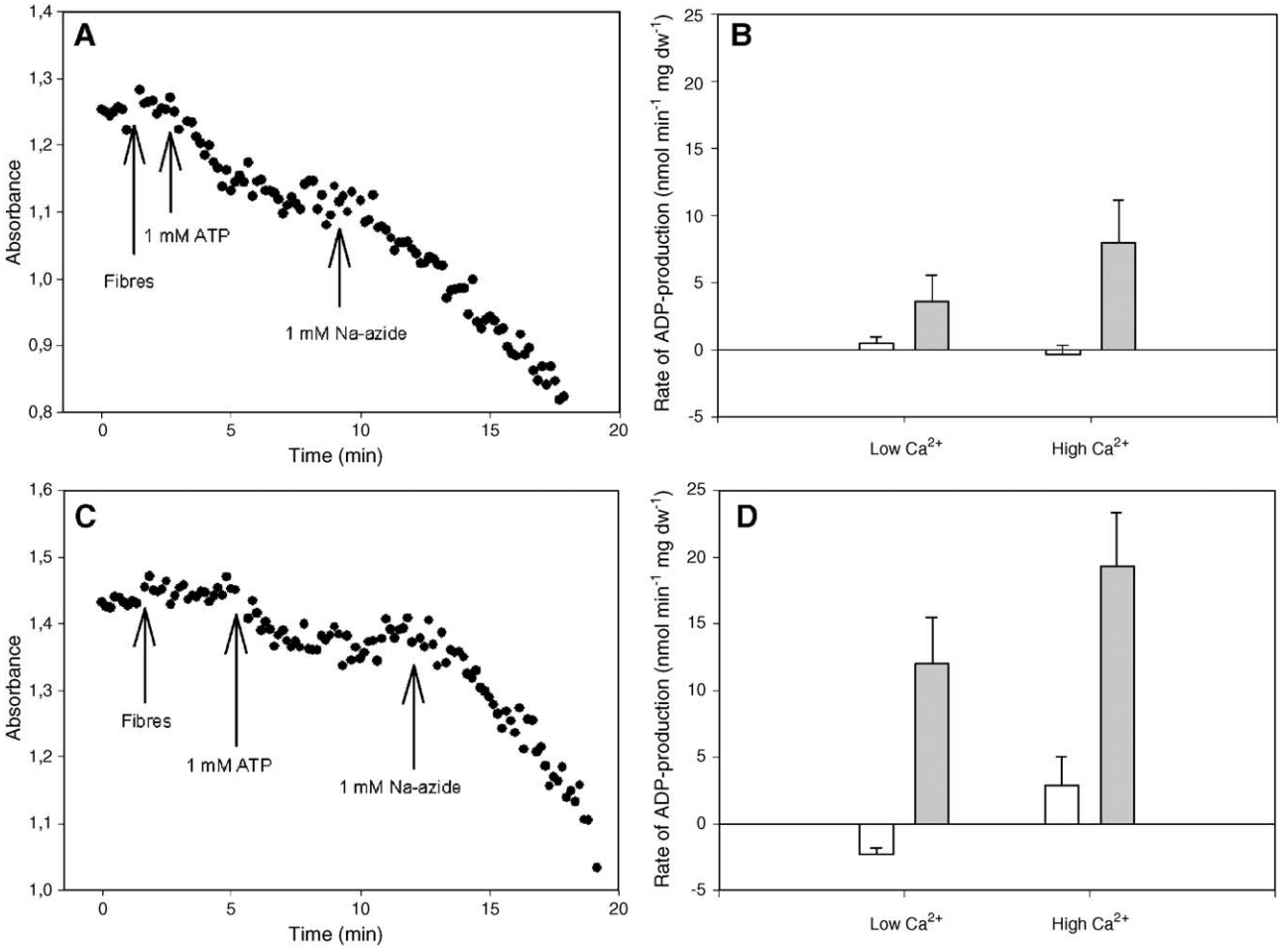

Fig. 4. Release of endogenously generated ADP from skinned fibres into the surrounding respiratory medium. The production of endogenous ADP from ATPases was initiated by the addition of 1 mM ATP to the respiratory medium. ADP-release was recorded spectrophotometrically as a function of time first in the absence and then in the presence of 1 mM Na-azide, which inhibits mitochondrial respiration. Panels A and C show representative traces of absorbance as a function of time. Panels B and D show the averaged rates of ADP-release calculated from the absorbance change during 200 s immediately before (white bars) and after (grey bars) addition of Na-azide. Results are mean±S.E.M. for three fibre preparations from rainbow trout (A and B) and Atlantic cod (C and D), respectively.

respectively. Immediately after the addition of 1 mM ATP to the medium, there was a decrease in absorbance. This initial decrease was due to ADP contamination of ATP, as it was also observed upon addition of ATP in the absence of fibres (data not shown). Indeed, the decrease levelled off after 1–2 min and became close to zero. However, after addition of 1 mM Na-azide to block mitochondrial respiration, a steady decrease in absorbance was observed. Fig. 4B and D show the rate of ADP release for 200 s immediately before addition of 1 mM Na-azide and at steady state after addition of Na-azide.

3.5. Enzyme activities

The activity of key enzymes was recorded in tissue from some of the experimental animals. The results are shown in Table 3. The activities of citrate synthase (CS), adenylate kinase (AK) and creatine kinase (CK) were higher in trout than in cod, suggesting that both the aerobic metabolic capacity and the energy buffering capacity were higher. The anaerobic capacity in terms of pyruvate kinase activity only tended ($P=0.0601$) to be

Table 3
Activity of key metabolic enzymes (μmol min^{-1} g ww^{-1}) in whole tissue homogenate, and the fraction of CK activity in skinned fibres relative to that in whole tissue homogenate (%; calculated by relating the CK activity to the CS activity in skinned fibres and whole tissue homogenate)

	CS	AK	CK	CK skinned fibres (%)	PK	LDH	PIR
Trout	24±1	61±5	95±4	12±2	52±3	144±25	1.27±0.10
Cod	12±2***	4±1***	30±5***	7±1	66±5	309±35**	1.58±0.19

**$P<0.01$ and ***$P<0.001$ significant difference between the two species.
The enzyme activities were recorded spectrophotometrically under optimal conditions. CS, citrate synthase; AK, adenylate kinase; CK, creatine kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase. LDH was furthermore recorded at a high (10 mM) pyruvate in order to obtain the pyruvate inhibition ratio, PIR, in terms of the ratio of the LDH activity at 0.35 mM pyruvate to that at 10 mM. Results are mean±SEM from five rainbow trout and five Atlantic cod.

higher in cod than in trout. LDH activity was higher in cod than in trout, but the pyruvate inhibition ratio was similar in the two species and above one, which suggests prevalence of the H-type of LDH [26].

4. Discussion

The present study resulted in two important findings

1. In skinned myocardial fibres from rainbow trout and Atlantic cod, activation of PK in the cytosolic bulk phase did not lower respiration rate more than 40%. This suggests that the intracellular environment is compartmented in such a way that ADP is channelled from ATPases to mitochondria without being released to the cytosolic bulk phase. In perspective, this suggests that compartmentation of the intracellular environment is a general design of cardiomyocytes and not limited to endotherms or species with a CK system coupled to mitochondrial respiration.
2. In trout heart, activation of PK abolished the stimulatory effect of creatine on respiration. This was surprising because previous studies have shown that activation of CK increases the apparent ADP affinity [16,19]. This may suggest that in the present experiment, the stimulatory effect of creatine on respiration was due to activation of CK outside the mitochondria.

4.1. Channelling of ADP in cardiac fibres from rainbow trout and Atlantic cod

The mitochondrial apparent $K_{M\ ADP}$ in skinned myocardial fibres from rainbow trout and Atlantic cod (Table 1) was more than one order of magnitude higher than the value reported for isolated mitochondria from fish oxidative muscle [30,31]. This is in agreement with a previous study [16] and suggests that diffusion of ADP from the respiratory medium to the mitochondria in skinned fibres is restricted. Additionally, in both trout and cod, the apparent ADP affinity was higher for endogenous ADP generated by ATPases upon stimulation with 1 mM ATP than for exogenous ADP added to the respiratory medium (Fig. 2). This was also found in a previous study on trout [19] and suggests that endogenous ADP generated by ATPases is preferentially channelled to mitochondria rather than being released to the respiratory medium surrounding the skinned fibres.

In the present experiments, the intracellular ADP channelling in rainbow trout and Atlantic cod cardiomyocytes was approached by a competitive assay in which added PK was activated to compete with mitochondria for endogenous ADP generated by ATPases [2]. The total ADP consumption capacity of the PK in solution and thus the cytosol phase of the skinned fibres exceeded that of the mitochondria by at least a factor of 100 (see Materials and methods). Therefore, if the cytosol was without compartmentation, and ADP generated by ATPases was equally accessible to PK and mitochondria, PK would be expected to consume almost all the ADP and to more or less abolish ATP-stimulated respiration. However, at low Ca^{2+} activity, activation of 10 IU/ml PK reduced $V_{1\ mM\ ATP}$ by only 40% in trout and 26% in cod (Fig. 3). This reduction of respiration rate is similar to that observed in skinned rat cardiac fibres [2] and suggests that PK did not have access to a large fraction of the ADP produced by intrinsic, Ca^{2+}-independent ATPases. Instead, this ADP fraction was channelled directly to the mitochondria.

This interpretation of the results is based on the assumption that PK in the surrounding medium is equilibrated with the cytosol of the skinned fibres. According to a previous experiment on trout skinned fibres, the applied saponin skinning releases about 85% of the LDH activity [19]. Thus, the perforation of the sarcolemma should allow enzymes to enter into the preparation. Furthermore, PK was present in the respiration medium at the beginning of the experiments and thus allowed at least 5 min for equilibration, which should be more than sufficient [20]. Finally, if more PK entered the cytosol during the experiments, the respiration rate would be expected to slowly decrease. However, in Fig. 1, the slope of the curve showing $[O_2]$ in the chamber as a function of time is linear. We are therefore confident that PK in the medium was fully equilibrated with the accessible cytosol of the skinned fibres.

The experiments were also carried out at a high Ca^{2+} activity, pCa = 5.41, which has been shown to maximally activate the myosin-ATPase in Triton-X-treated preparations of trout heart at 10 °C [22]. It might be expected that Ca^{2+}, which is known to activate certain mitochondrial dehydrogenases, would increase the respiration rate [32]. However, we observed a marked decrease in $V_{ADP\ max}$ (Table 1). The reason for this is unclear. A similar effect has also been found in skinned rat cardiac fibres and was supposed to relate to the deformation occurring during hypercontraction of the fibres rather than to mitochondrial Ca^{2+} overload [33].

At the high Ca^{2+} activity, compartmentation seemed to be accentuated inasmuch as activation of PK had no effect on mitochondrial respiration (Fig. 3). Thus, all the ADP generated by ATPases was channelled to the mitochondria without being accessible to PK. This effect of Ca^{2+} is in agreement with the finding that the apparent affinity for both exogenous and endogenous ADP increased with an increase in Ca^{2+} activity (Table 1 and Fig. 2). It is possible that high Ca^{2+} abolishes the effect of PK by triggering structural modifications, which may increase the efficiency of ADP-channelling [33]. Such modifications may involve (1) a reduction of the distance between ATPases and mitochondria, and/or (2) a tightening of diffusion barriers that increases channelling.

The hypothesis of a channelling of ADP between ATPases and mitochondria was further supported by our spectrophotometric observation that endogenous ADP generated by ATPases was not released from the fibres unless mitochondrial respiration was inhibited with Na-azide (Fig. 4). This has also been found in skinned rat cardiac fibres [2].

4.2. Pyruvate kinase abolishes creatine-stimulated respiration in skinned rainbow trout cardiac fibres

The experiments with creatine provided some unexpected results. In cod, creatine did not affect $V_{1\ mM\ ATP}$ (Fig. 3B), which is in accordance with a previous investigation [16]. In

trout, creatine increased $V_{1\ mM\ ATP}$ by 184% and 58% at low and high Ca^{2+} activity, respectively (Fig. 3A). This is in agreement with previous investigations showing that creatine increases the apparent ADP affinity [16], and that ADP produced by CK seems to be transported to the mitochondria rather than out of the skinned fibres [19]. Therefore, it was surprising that activation of PK abolished the stimulatory effect of creatine (Fig. 3A): in studies of skinned mouse cardiac fibres [34] and isolated rat cardiac mitochondria [35], ADP produced by Mi-CK in the intermembrane compartment is inaccessible to PK. on basis of the present finding, it might be speculated that rainbow trout in contrast to mammalian cardiomyocytes do not express a mitochondrial form of CK to any significant extent, and that the stimulatory effect of creatine observed in previous and in the present experiments is due to an increased ADP feedback from cytosolic CK. Indeed, creatine also increases the apparent ADP affinity in skinned cardiac fibres from Mi-$CK^{-/-}$ knockout mice [36]. However, it is important to note that cardiomyocytes from Mi-$CK^{-/-}$ mice and rainbow trout differ from wild-type mouse and rat cardiomyocytes in as much as creatine does not bring down the apparent $K_{M\ ADP}$ to the range of 20–50 μM recorded for isolated mitochondria [30,37]. Thus, it is possible that stimulation of cytosolic CK may increase the apparent ADP affinity to some extent, but Mi-CK functionally coupled to ANT is needed to lower the apparent $K_{M\ ADP}$ down to levels recorded for isolated mitochondria. However, these are only speculations, which open the way for further investigations on isolated mitochondria [35].

It may seem intriguing that creatine stimulates respiration in skinned trout cardiac fibres only by stimulation of cytosolically accessible CK, and it might be asked whether the fibres were poorly separated so that the high apparent $K_{M\ ADP}$ in our skinned fibre preparation was due to a large diffusion distance from the surrounding medium to the mitochondria inside the fibres. However, diffusion distance cannot explain the low ability of PK to compete with mitochondrial respiration for endogenous ADP (Fig. 3), and the spectrophotometric observation that ADP was not released from the skinned fibre preparation unless mitochondrial respiration was inhibited with azide (Fig. 4). Additionally, it cannot explain the previous observation that $K_{M\ ADP}$ increases when temperature decreases [16]: a temperature decrease should reduce the importance of diffusion distance and decrease $K_{M\ ADP}$, because temperature affects diffusion flux ($Q_{10} \sim 1.3$) less than it affects metabolism ($Q_{10} \sim 2$) [7,8]. Therefore, the high apparent $K_{M\ ADP}$ in skinned cardiac fibres from rainbow trout and Atlantic cod should in all likelihood be due to compartmentation.

4.3. Compartmentation is a general feature of cardiomyocytes

It is interesting to compare the functional characteristics of mitochondria and the CK system in cardiomyocytes of species with different metabolic demands determined by the behaviour of the animal. This may give further insight into the cause and functional significance of compartmentation and CK.

This study has provided further evidence that skinned cardiac fibres from rainbow trout and Atlantic cod resemble those of mammals as to the existence of compartments that are separated from the cytosolic bulk phase [1,3,38]. Intracellular compartmentation thus seems to be a general feature of cardiomyocytes occurring in both endo- and ectothermic species irrespective of metabolic rate, working temperature, intracellular membrane density, and functional significance of the CK system.

The role of CK, however, varies between species, and this may relate to the metabolic demand. As an indication of the metabolic pattern, we recorded the activities of enzyme markers in trout and cod myocardia (Table 3). The higher CS activity in trout myocardium (Table 3) suggests that it relies on aerobic energy production to a greater extent than cod myocardium. This is in agreement with the finding that $V_{ADP\ max}$ tended to be higher in trout than in cod (Table 1). The aerobic capacity as indicated by the activity of cytochrome oxidase in whole tissue homogenate is similar in trout and rat when recorded at the same temperature [39]. As to the behaviour, cod is slow moving with only short bursts of activity in critical situations, whereas trout is relatively active. However, even at high sustained activity, the metabolic rate of trout will never reach that of a mammal because of differences in intrinsic metabolic rate and working temperature. The importance of CK in the regulation of respiration is negligible in cod, and significant in trout, but to a lesser extent that in mammals. This could suggest that in cardiac fibres the importance of CK in regulating mitochondrial respiration is related to the level and sustainability of energy demand. A similar idea was proposed by Ventura-Clapier et al. [17].

In conclusion, intracellular compartmentation seems to be a general design of cardiomyocytes, which rely on a very efficient feedback of ADP from ATPases to mitochondria. In mammalian cardiomyocytes, CK is tightly coupled at a molecular level to ATPases and ANT in the mitochondria and may enhance this feedback. However, in ectothermic vertebrates, these requirements may be less and the CK system less elaborated.

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