Volume 256, number 1,2, 97–100 FEB 07679 October 1989

Reversible modulation of the mitochondrial ATP synthase with energy demand in cultured rat cardiomyocytes

Anibh M. Das and David A. Harris

Department of Biochemistry, University of Oxford, South Parks Rd, Oxford OX1 3QU, England

Received 14 July 1989; revised version received 21 August 1989

The ATP synthase capacity of rat heart myocytes can be measured in sonicates of cultured cardiomyocytes. In these cells, transitions in ATP synthase capacity occur on changing to the anoxic or uncoupled state (drop in ATP synthase capacity of over 40%) or on electrically stimulating the cells to contract (rise of 70%). These changes occur rapidly (half time less than 1 min) and are completely reversed on returning to the original conditions. It is proposed that mitochondria in vivo are directly regulated at the level of the ATP synthase. The naturally occurring inhibitor protein from mitochondria may be responsible for this regulation.

Mitochondria; Cardiomyocyte; ATP synthase; ATPase inhibitor protein; Metabolic regulation; Anoxia

1. INTRODUCTION

The mitochondrial ATP synthase (F_1-F_0-ATPase) (for a review see [1]) is responsible for the bulk of ATP synthesis in (aerobic) heart tissue. As energy demand changes, the flux through this enzyme must also change so that ATP synthesis matches ATP utilisation. In heart, 5–10-fold increases in flux are not uncommon.

It is generally believed that the mitochondrial ATP synthase responds simply to ADP levels – that it has a high capacity relative to its turnover and, as ADP levels rise, the enzyme achieves a higher degree of saturation (and thus faster turnover). This view, however, has been questioned. First, the ATP synthase in heart mitochondria has been shown to be a potential control site since it has a significant control strength at physiological rates of ATP synthesis [2]. Secondly, a variety of regulatory elements have been shown to act at the level of the ATP synthase (for reviews see [3,4]).

In particular, a regulator protein, IF_1, has been identified as occurring naturally in the mitochondria of a variety of mammals [5–7]. This protein inhibits the ATP synthase (in both the synthetic and hydrolytic directions) in a potential- and ATP-dependent manner [8,9] and its properties in submitochondrial vesicles from ox heart have been well characterised [10]. This protein would seem to be a good candidate for a regulator of the ATP synthase in vivo.

Work by Rouslin [11,12] has supported the idea that this protein may inhibit the F_1-F_0-ATPase of heart mitochondria in some animals during periods of ischaemia, although no effect was found in the rat. We show here that modulation of ATP synthase activity, consistent with its regulation in vivo by such a regulator protein, does indeed occur in intact rat cardiac myocytes. Further, such regulation applies in conditions other than ischaemia, such as quiescence, stimulated contraction, anoxia and uncoupling.

Correspondence address: D.A. Harris, Department of Biochemistry, University of Oxford, South Parks Rd, Oxford OX1 3QU, England

Abbreviations: F_1, soluble portion of the mitochondrial ATP synthase complex; IF_1, naturally occurring inhibitor protein of F_1; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. MATERIALS AND METHODS

Calcium-tolerant cardiomyocytes were obtained from the hearts of female Wistar rats by the method of Powell et al. [13]. Freshly isolated myocytes were cultured using the rapid cell attachment procedure of Piper et al. [14]. Briefly, polystyrene culture dishes (Falcon, type 3002) were preincubated overnight at 37°C with 4% (v/v) fetal calf serum (FCS) in medium 199 (M199). Isolated cells, from the above procedure, were diluted in 4% FCS/M199 to about 1×10^5 cells/ml, 3 ml added to the precoated culture dishes and incubated for 4 h at 37°C. Under these conditions, live cells attached to the plates and dead cells could be removed by aspiration.

After attachment, the proportion of rod shaped (i.e. viable) cells was >95% and cellular ATP was 37.9 nmol/mg protein, in agreement with [14]. Cells in culture were stable for several days. Cultured cells, obtained after an attachment period of 4 h, were used in the experiments described below.

Petri dishes with attached myocytes (typically 1×10^5 cells per dish) were washed twice with buffer I [25 mM Hepes, 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4 (NaOH)], and incubated in 2 ml of this buffer with appropriate additions (see text). For ATPase assay, buffer I was replaced by buffer II [20 mM Hepes, 1 mM $MgCl_2$, 2 mM EGTA, pH 7.0 (NaOH)], and the cells immediately sonicated 3 × 10 s at 25°C using an MSE probe sonicator (150 W) at 10 μm amplitude. (This neutral, low salt buffer was chosen for cell disintegration as IF_1 is displaced from F_1 at high salt or high pH.) ATPase activity and NADH oxidase activity were measured as previously [10] except that 2 mM EGTA replaced EDTA in the reaction medium. Under these conditions, >90% of the measured ATPase was mitochondrial as shown by sensitivity to oligomycin and azide. Neither FCCP nor carboxyatractylate affected the ATPase activity measured, confirming that the mitochondrial membranes were fully broken. Protein was measured according to [15].

Collagenase (Worthington Type 1, *Cl. histolyticum*) was obtained from Lorne Laboratories (Twyford, England). For cell isolations, purified bovine serum albumin (Behringwerke, Marburg, FRG) or Pentex bovine albumin, fraction V (Miles Laboratories, Naperville, IL, USA) was used. M199 and fetal calf serum were obtained from Boehringer (Mannheim, FRG).

3. RESULTS

As previously [10], we have used ATP hydrolysis at saturating [ATP] as a measure of ATP synthase capacity, since the synthase works in both hydrolytic and synthetic directions. If the ATP synthase, in the cells, is activated (and this activated state is stable to sonication, etc.), submitochondrial vesicles derived from these cells will have an increased ATPase activity.

Table 1 shows that, as the metabolic state of myocytes is changed, the capacity of their mitochondrial ATP synthase changes. When cells are shifted from the quiescent, oxygenated state to an oxygen-free buffer (100% N_2, anoxia), the ATP synthase capacity falls from 100% (3.7 μmol/min per mg protein) to 63%. This appears to be related to the energy state of the mitochondria, since incubating the cells with uncoupler (table 1) or cyanide (data not shown) prior to sonication leads to an even greater drop in synthase capacity. (Note that addition of uncoupler to the ATPase assay medium does not affect turnover rate: these observations relate to effects on the cells themselves.) In contrast, electrical stimulation of these myocytes (leading to contraction) leads to a 70% increase in mitochondrial ATP synthase activity. Thus, when energy demand in the cell increases, the ATP synthase is switched on.

Table 1

ATP synthase capacity in cultured cardiomyocytes exposed to metabolic transitions

Treatment of cells	ATPase activity (μmol/min per mg protein)	NADH oxidase activity (μmol/min per mg protein)
O_2	3.7 ± 0.3	0.10 ± 0.01
N_2	2.3 ± 0.1	0.11 ± 0.02
FCCP	1.5 ± 0.1	0.11 ± 0.01
Stimulation	6.5 ± 0.4	0.13 ± 0.02

Cultured myocytes (1×10^5 cells/plate) were prepared, and incubated in 2 ml buffer I (see section 2) gassed with 100% O_2. Their mitochondrial ATPase capacity was measured after sonication, as described in section 2. Where indicated, O_2 was replaced with 100% N_2 for 15 min, or the cells were incubated with 2 μM FCCP, or stimulated electrically (20 V/cm at 3 Hz, 0.5 ms per pulse) for 2 min. Results are expressed as mean ± SD ($n > 7$)

To eliminate the possibility that these transients reflect changes in mitochondrial recovery rather than activity, NADH oxidase activity was monitored in the same extracts as a mitochondrial marker. Table 1 shows that this activity was constant at around 0.1 μmol/min per mg irrespective of which pretreatment was involved. It was concluded that mitochondrial recovery was constant, and that the observed differences did indeed reflect changes in ATP synthase activation.

These changes in ATP synthase capacity occurred rapidly and were rapidly reversed. This is demonstrated for electrically stimulated cells in fig.1. The half time for activation following commencement of stimulation was about 40 s, and after cessation of stimulation, the mitochondria

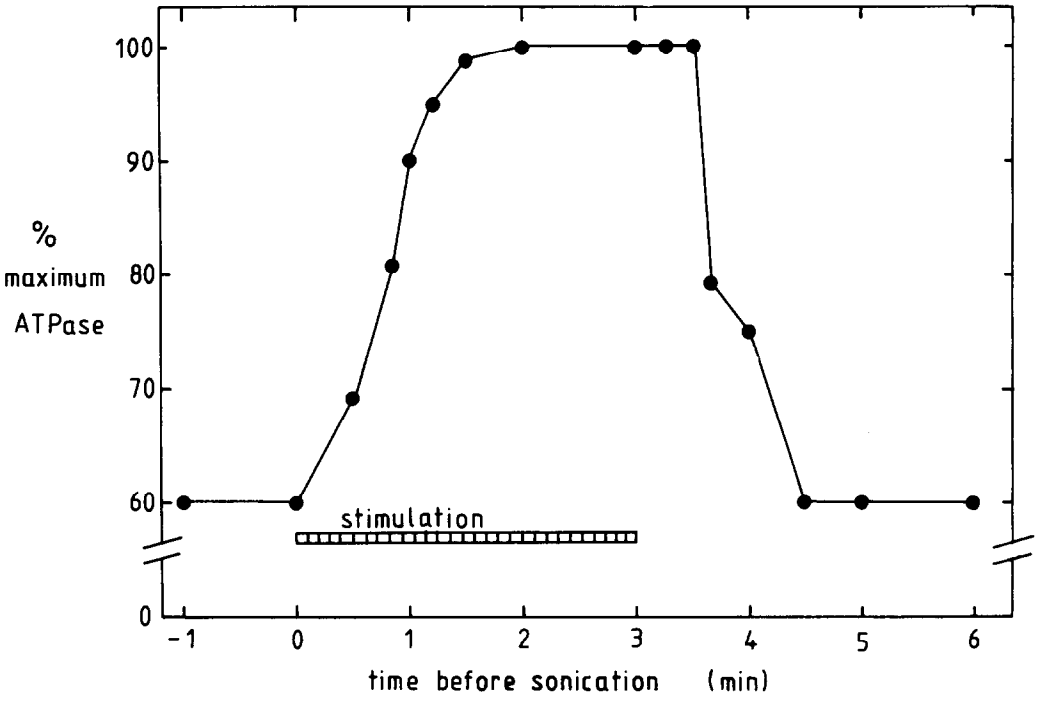

Fig.1. Time course of changes in ATP synthase capacity. Cultured cardiomyocytes were incubated in buffer I (see section 2) and their mitochondrial ATPase activity measured after electrical stimulation as described in table 1, except that samples were taken after various periods of stimulation, or after cessation of stimulation as indicated. 100% = 6.5 μmol/min per mg protein (see table 1).

reverted to their original state within about 2 min. In fact, the changes themselves probably took place faster than this, but there appeared to be an initial lag. This is clearly visible in the inactivation profile, and may also be present during activation (fig.1). This may represent the creation of, and recovery from an 'energy debt' within the cells during contraction. Similarly rapid inactivation and recovery is seen in the oxygenated-anoxic-oxygenated transition (data not shown).

4. DISCUSSION

We show above that the mitochondrial ATP synthase, in cultured rat heart cells, changes in capacity with their metabolic state. During electrical stimulation, the synthase is switched to a more active state; when oxygen is removed, or the system uncoupled (such that the ATP synthase could hydrolyse ATP in vivo), the enzyme is switched off (table 1). These changes are rapidly reversible inside the cells (fig.1), as would be required of a regulatory mechanism. Down regulation of heart mitochondrial ATPase in ischaemia has been demonstrated by Rouslin in species other than rat [11,12], and it has been proposed that this inhibition may limit futile hydrolysis of ATP in ischaemia. However, the present findings are the first demonstration that the mitochondrial ATP synthase in any species can be regulated in vivo in other than pathological situations, and that this regulation is rapid and reversible.

Since the regulated states of the ATP synthase are stable to dilution of broken mitochondria into a large volume of assay buffer, a rapidly reversible, allosteric mechanism with a small molecular regulator is ruled out. The results are, however, compatible with the participation of a protein regulator, the mitochondrial ATPase inhibitor protein IF_1. This protein binds to the F_1 portion of the mitochondrial ATP synthase, inhibiting its (synthetic and hydrolytic) activities [9]. It has been shown to regulate the activity of the F_1-F_0-ATP synthase in ox heart submitochondrial vesicles [10]

and a homologous protein has been demonstrated in the rat [6]. It is thus proposed that mitochondrial ATP synthase in rat heart in vivo may be regulated by varying its association with a naturally occurring inhibitor protein, IF_1.

Some of the properties of the regulation observed are consistent with the known properties of the F_1-IF_1 interaction from ox heart mitochondria – for example the time course of the change and the promotion of inhibition at low membrane energisation [9,10]. The effect of electrical stimulation cannot be so simply explained, however, and must await further experimentation. However, it is possible that intramitochondrial Ca^{2+} might affect the F_1-IF_1 interaction in this situation.

Whatever the mechanism, these results run counter to the generally accepted view of the regulation of ATP synthesis in vivo. Typically, it is believed that the ATP synthase is not regulated (its capacity is unchanged) with varying metabolic conditions, and that it responds to increasing ATP demand simply by responding to increasing ADP levels – the normal response of an (unsaturated) enzyme to increasing substrate. This view cannot account for the changes in ATP synthase capacity observed above: these clearly demonstrate that some direct regulation of the ATP synthase does occur within intact cells.

Acknowledgement: This work was supported by the British Heart Foundation.

REFERENCES

[1] Senior, A.E. (1988) Physiol. Rev. 68, 177–231.
[2] Doussiere, J., Liget, E., Brandolin, G. and Vignais, P.V. (1984) Biochim. Biophys. Acta 766, 492–499.
[3] Harris, D.A. (1984) in: H^+-ATPase (ATP Synthase): Structure, Function, Biogenesis (Papa, S. et al. eds) pp.387–395, Adriatica Editrice, Bari.
[4] Chernyak, B.V. and Koslov, I.A. (1986) Trends Biochem. Sci. 11, 32–36.
[5] Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762–3769.
[6] Cintron, N.M. and Pedersen, P.L. (1979) J. Biol. Chem. 254, 3439–3443.
[7] Yamada, E.W., Huzel, N.J. and Dickison, J.C. (1981) J. Biol. Chem. 256, 10203–10207.
[8] Gomez-Puyou, A., Tuena de Gomez-Puyou, M. and Ernster, L. (1979) Biochim. Biophys. Acta 547, 252–257.
[9] Harris, D.A., Von Tscharner, V. and Radda, G.K. (1979) Biochim. Biophys. Acta 548, 72–84.
[10] Lippe, G., Sorgato, M.C. and Harris, D.A. (1988) Biochim. Biophys. Acta 933, 12–21.
[11] Rouslin, W. (1983) J. Biol. Chem. 258, 9657–9661.
[12] Rouslin, W. (1987) Am. J. Physiol. 252, H622–H627.
[13] Powell, T., Terrar, D.A. and Twist, V.W. (1980) J. Physiol. 302, 131–151.
[14] Piper, H.M., Probst, J., Schwartz, P., Hütter, J.F. and Spieckermann, P.G. (1982) J. Mol. Cell. Cardiol. 14, 397–412.
[15] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.