Intracellular calcium as a regulator of the mitochondrial ATP synthase in cultured cardiomyocytes

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The mitochondrial ATP synthase, in heart cells, must adjust its activity to meet the demands of the cells for energy. For many years, it has been held that the synthase is not regulated *per se*, but simply responds to the availability of its substrates, ADP, P_i and the transmembrane proton gradient. However, we have recently demonstrated that, within heart cells, the capacity of mitochondrial ATP synthase (its $V_{max.}$) is controlled. Rapid, reversible transients in this capacity, over a 4-fold range, were demonstrated in anoxia or inhibition of electron flow (ATP synthase capacity falls), or under electrical stimulation (capacity rises) [1, 2].

Ca^{2+} ions are known to act as a messenger between cytoplasm and mitochondria, and are involved in the regulation of some Krebs cycle dehydrogenases [3]. We thus investigated the possibility that Ca^{2+} might be involved in the regulation of the ATP synthase itself.

Ca^{2+} tolerant myocytes were isolated from male Wistar rats (250–300 g) and cultured using the rapid attachment method of Piper *et al.* [4]. After 4 h of incubation, more than 95% of the cells were intact and quiescent, but capable of beating on electrical stimulation. Cells were then incubated in 2 ml of 25 mM-Hepes, 110 mM-NaCl, 2.6 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 1 mM-$CaCl_2$, 10 mM-glucose, pH 7.4 (NaOH), at 37°C, with additions as indicated, before measurement of mitochondrial ATPase activity (taken as an indication of synthase capacity). Detailed characterization of the cultured cells and measurement of mitochondrial ATPase (in a diluted cell sonicate), were as described elsewhere [2].

Table 1 shows that, when cardiomyocytes are stimulated electrically, they contract (in synchrony with the stimulation at 10 Hz), and their mitochondrial ATPase activity rises to about 180% of the control value (measured on non-contracting cells). This is consistent with the view that ATP synthase capacity rises to meet increased energy demand by contracting cells. This change is rapid (half-time < 1 min) and reversed on cessation of stimulation (data not shown).

To investigate the role of Ca^{2+} in this process, Ca^{2+} was withdrawn from the medium. In this case, both contraction and the increase of the ATP synthase capacity were abolished (Table 1). Further, verapamil (which blocks Ca^{2+} entry into cells) and Ruthenium Red (which blocks Ca^{2+} entry into mitochondria from the cytoplasm) also abolish the increase in mitochondrial ATPase at levels which do not block contraction in the short term. These observations indicate that it is the $[Ca^{2+}]$, and in particular the intramitochondrial $[Ca^{2+}]$, rather than contraction *per se*, which modulates the ATP synthase.

Addition to the cells of positive inotropic agents also has marked effects on the ATP synthase capacity. Both the β-agonist isoprenaline, and the phosphodiesterase III inhibitor SKF 94120 increased the mitochondrial ATPase of quiescent cells to about 200% and 120% of the respective value observed in the untreated cells. A further increase (to about 250%) was observed when the cells were stimulated to contract. Ruthenium Red abolished all these effects; in its presence, the ATPase remains at, or just below, the control value whether or not these inotropic agents are present. These observations suggest that the effects of these agents on mitochondrial ATP synthase are also mediated through intramitochondrial Ca^{2+} levels, rather than directly through cyclic AMP. A similar model has been proposed to explain the effects of these agents on the intramitochondrial dehydrogenases [3]. Interestingly, the inhibitor of a different class of phosphodiesterases, rolipram (10 μM) opposes the increase in mitochondrial ATP synthase capacity (Table 1). This may be the result of this (non-inotropic) drug affecting a different pool of cyclic AMP to SKF 94120; the existence of several pools of cyclic AMP in heart cells has previously been proposed [5].

It is notable that cells treated with positive inotropic agents do not show spontaneous contracture despite an apparently raised mitochondrial (and presumably cytoplasmic) $[Ca^{2+}]$. This indicates that their cytosolic $[Ca^{2+}]$ must be lower than the peak concentration in contracting cells, despite a higher ATP synthase activity. This can be explained if the relatively sluggish Ca^{2+} transport system of mitochondria leads to the intramitochondrial $[Ca^{2+}]$ (which controls the synthase capacity) reflecting the time averaged, rather than the instantaneous, cytoplasmic concentration. Further work is in progress to test this model.

We conclude: (*a*) that the mitochondrial ATP synthase is regulated *in vivo*, by a non-allosteric mechanism and (*b*) that a major element in this regulation is the intramitochondrial $[Ca^{2+}]$. However, it is not clear how Ca^{2+} affects the ATP synthase; assay conditions (which include dilution) preclude an allosteric mechanism, while protein phosphorylation, such as modulates mitochondrial pyruvate dehydrogenase [3], does not apparently influence the ATP synthase. The only regulatory mechanism known to affect the mitochondrial ATP synthase is that involving an inhibitory protein which binds reversibly to it [6]. If, and how, the interaction between this protein and the ATP synthase can be affected by Ca^{2+} remains to be established.

Table 1. *Responses of mitochondrial ATP synthase capacity to cellular energy demand*

Cultured cardiomyocytes were incubated for 10 min at 37°C with the drugs indicated. Electrical field stimulation of the cells was then carried out for 2 min using a square-wave generator (20 V/cm, 0.5 ms/pulse, frequency 10 Hz). The bathing medium was then rapidly (< 10 s) changed to 20 mM-Hepes, 1 mM-$MgCl_2$, 2 mM-EGTA, pH 7.0 (NaOH), and the cells sonicated for two periods of 15 s. Oligomycin-sensitive ATPase activity was measured on the sonicate [2]. +RR indicates incubation of the cells with Ruthenium Red (3 μg/ml) in addition to the drug named. Results are given as mean ± S.E.M. ($n > 6$). n.d., Not determined.

	Oligomycin-sensitive ATPase ($\mu mol^{-1}\ min^{-1}\ mg$)			
	Quiescent cells		Stimulated cells	
Addition	−RR	+RR	−RR	+RR
None	3.5 ± 0.1	3.2 ± 0.1	6.3 ± 0.1	3.4 ± 0.1
Ca^{2+} omitted	3.5 ± 0.1	n.d.	3.5 ± 0.2*	n.d.
Verapamil (10^{-8} M)	3.2 ± 0.1	n.d.	3.2 ± 0.1	n.d.
Isoprenaline (10^{-5} M)	7.0 ± 0.1	2.8 ± 0.1	8.6 ± 0.1	3.4 ± 0.1
SKF 94120 (10^{-5} M)	4.2 ± 0.1	3.5 ± 0.1	8.3 ± 0.3	3.1 ± 0.1
Rolipram (10^{-5} M)	n.d.	n.d.	4.6 ± 0.1	n.d.

*Indicates abolition of contraction.

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A T-cell protein which recognizes a palindromic DNA sequence in the negative regulatory element of the HIV-1 long terminal repeat with homology to steroid/thyroid hormone receptor binding sites

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Expression from the human immunodeficiency virus 1 long terminal repeat (HIV-1 LTR) is known to be regulated by host cell parameters such as T-lymphocyte activation [1–3], lymphokine action [4, 5] and macrophage differentiation [6]. These events have the potential to influence critically the progress of disease in infected individuals. A number of cellular proteins capable of binding to the HIV-1 LTR have been identified [1–3, 7, 8], and one of these, NFKB, binds to the enhancer region of the LTR and increases viral transcription in response to several stimuli [1, 4–6]. In contrast, however, the region of the LTR upstream of −278 (relative to the start of transcription) has been identified as a negative regulatory element (NRE), the removal of which increases LTR expression in the Jurkat T cell line [9], and which may therefore play a role in the maintenance of HIV latency. The cellular poteins which interact with the NRE are clearly of interest as potential transcriptional repressors. We have defined the two major protein binding sites within the NRE, both of which bind previously undescribed human T-cell proteins. One of these sites has sequence homology with, and binding characteristics of, a steroid/thyroid hormone response element [10], but is distinct from previously described binding sites of this type.

To characterize proteins interacting with the upstream NRE of the HIV-1 LTR, we carried out DNAase 1 footprint analysis using a fragment containing bases −456 to −300 from the HXBH2 LTR and nuclear extract from Jurkat T cells. This fragment was selected to contain the NRE as defined in both resting and activated T cells [9], but lacking the binding site for the potential positive regulatory factor NFAT1, present in such cells [2, 3]. We detected two footprints within this fragment which are located at −379 to −361 (site A), and −350 to −327 (site B). Each of these footprints was specifically removed with a 30 bp oligonucleotide spanning the footprint site, while competition with both sites resulted in a footprint indistinguishable from that in the absence of nuclear extract. Unrelated oligonucleotides had no effect on the footprint at either site.

DNA mobility shift analysis of a site B oligonucleotide (Fig. 1(*b*), track A) revealed a single major DNA–protein

Abbreviations used: HIV, human immunodeficiency virus; LTR, long terminal repeat; NRE, negative regulatory element; ERE, oestrogen response element; TRE, thyroid hormone response element; GRE, glucocorticoid response element.

(*a*)

Site B HIV-1 LTR	AGG**GGTCA**GATATCCAC**TGACC**TTC
ERE	TCA**GGTCA**CAG**TGACC**TGA
TRE	TAA**GATCA**GGACG**TGACC**GCA
GRE	TCA**GAACA**CAG**TGTTC**TGA

(*b*)

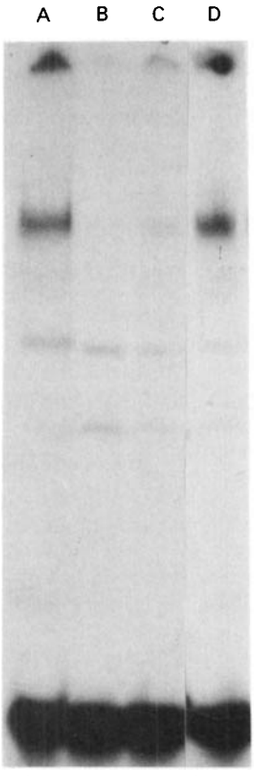

Fig. 1. *Characterization of site B*

(*a*) Sequence of site B in the negative regulatory element of the HIV-1 LTR compared with binding sites for previously identified steroid/thyroid hormone receptors, with palindromic regions in bold type. (*b*) DNA mobility shift assay with labelled site B oligonucleotide and Jurkat T cell extract and no competitor (track A), site B oligonucleotide (track B), ERE (track C) and GRE (track D). Methods: 10 fmol of reverse transcriptase labelled oligonucleotides were incubated with 8 μg of Jurkat extract and competitor (sequence shown in panel A) in 100-fold molar excess.