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Adenine nucleotides regulate Ca^{2+} transport in brain mitochondria

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Adenine nucleotides (ADP > ATP) greatly enhance Ca^{2+} uptake and retention in rat brain mitochondria. In the presence of both spermine and ADP, brain mitochondria sequester Ca^{2+} down to cellular free Ca^{2+} levels, suggesting a role for mitochondria in modulating Ca^{2+} cycles in brain cells. Analysis of the effects of various inhibitors on Ca^{2+} uptake and efflux suggest that locking the ADP/ATP translocator in its M-state stimulates electrogenic Ca^{2+} uptake and, to a lesser extent, inhibits Ca^{2+} efflux. It is suggested that this effect is due to a modulation of the surface charge on the M-side which enhances Ca^{2+} dissociation from the carriers.

Ca^{2+}; Adenine nucleotide; Spermine; Mitochondria

1. INTRODUCTION

Calcium transport in mitochondria is catalyzed by several Ca^{2+}-specific transport systems. The most active system is the electrogenic Ca^{2+} carrier which mainly catalyzes Ca^{2+} uptake driven by the membrane potential. Mitochondria also contain a Ca^{2+}-Na^{+} exchange carrier which catalyzes Ca^{2+} efflux and another system which catalyzes Na^{+}-independent Ca^{2+} efflux [1]. The balance of the activities of these systems determines the Ca^{2+} 'set-point', i.e. the external free Ca^{2+} concentration at which net transport vanishes [2], and also the Ca^{2+} concentration in the mitochondrial matrix which controls the rate of respiration [3]. Studies with isolated liver and heart mitochondria have suggested that the Ca^{2+} set-point for mitochondria in a physiological medium is well above the physiological range of Ca^{2+} [1,2]. However, it was recently demonstrated that polyamines greatly enhance the ability of liver mitochondria to sequester Ca^{2+} over the physiological range [4]. The ability of isolated brain mitochondria to lower external Ca^{2+} within the physiological range has not been studied as extensively, but a recent study demonstrated that in brain mitochondria as well, polyamines enhance Ca^{2+} sequestration [5]. Here, we report the ability of rat brain mitochondria to sequester Ca^{2+} within the physiological concentration range, and in particular the crucial role of adenine nucleotides in regulating this process. These results suggest that mitochondria could play an important role in regulating cellular Ca^{2+} levels.

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2. MATERIALS AND METHODS

Rat brain 'free' mitochondria were prepared, essentially as described by Lai and Clark [6]. Ca^{2+} transport was measured either with Arsenazo III or with a Ca^{2+} electrode. The absorption difference (685–675 nm) of Arsenazo III was recorded on an Aminco DW-2A double-beam spectrometer. Free Ca^{2+} was estimated from calibration curves constructed from titration of Arsenazo III with Ca^{2+}. Separate calibration curves were constructed for each reaction medium [7]. An Orion Ca^{2+} electrode (93–20) was used to measure transport and set-point value at very low external Ca^{2+}. The electrode was calibrated essentially as described by Bers [8]. Bongkrekic acid was a generous gift from Dr A. Halestrap, University of Bristol and also from Dr J.A. Duine, Delft University of Technology. Other fine chemicals were from Sigma. All salts and sugars were of the highest analytical grade.

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3. RESULTS

In isolated brain mitochondria, adenine nucleotides determine the ability of mitochondria to sequester large amounts of Ca^{2+} and to retain a physiologically relevant set-point [2]. This is demonstrated in fig.1. Trace a shows a typical experiment in a medium which includes 10 μM ADP. Addition of succinate (which induces the formation of membrane potential) induced Ca^{2+} uptake. 30 nmol Ca^{2+} was sequestered and the set-point was approx. 0.2 μM. Another addition of 30 nmol Ca^{2+} raised the concentration to 3 μM which induced fast Ca^{2+} sequestration, reducing external Ca^{2+} again to a set-point of 0.2 μM, accumulating all the added Ca^{2+} (the total accumulated calcium was 120 nmol/mg protein). Addition of ruthenium red (RR), which inhibits the electrogenic carrier, induced Ca^{2+} efflux. Finally, EGTA was added to obtain the ΔA reading in the absence of Ca^{2+}. The free Ca^{2+} concentration at the set-points is obtained from the difference between the EGTA and steady-state reading, utilizing a calibration curve for Arsenazo III-Ca^{2+} association in this medium. Trace b shows the same experiment in the absence of added ADP. The initial uptake was slowed down, and the set-point was raised (0.4 μM). When additional Ca^{2+} was added, very little was taken up (<5 nmol) and the set-point was increased to 2.7 μM. As shown in trace c, atractyloside (ATR), a specific competitive inhibitor of the ADP/ATP translocator, completely inhibited the ADP effect. Although the ATP/ADP translocator mediates the effect of ADP on Ca^{2+} transport, net exchange of nucleotides is not required since another specific inhibitor of the translocator, bongkrekate, stimulates Ca^{2+} transport in a manner completely analogous to ADP even in the absence of added ADP (trace d). These seemingly paradoxical results can be rationalized on the basis of the known effects of these agents on the conformation of the ADP/ATP translocator. The carrier is known to exist in two major conformational states: M- and C-states. Both ADP and bongkrekate lock the carrier in the M-state, while atractyloside induces transformation to the C-state [9]. Thus, when the carrier is in the M-state, Ca^{2+} uptake and retention by brain mitochondria are enhanced, while the C-state inhibits both processes. Of many nucleotides that we tested (e.g. ADP, ATP, AMP, cAMP, cGMP, GDP, GTP, ITP), only ADP and ATP enhanced Ca^{2+} uptake and retention. ADP appeared to be considerably more effective than ATP, which is compatible with the affinity of the ATP/ADP translocator (not shown).

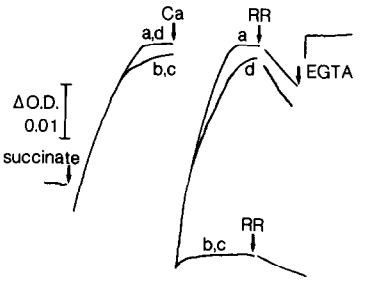

Fig.1. Effect of ADP on Ca^{2+} transport in rat brain mitochondria. The basic medium is composed of 0.2M manitol, 80 mM sucrose, 10 mM Tris-Cl, 5 mM Tris-P_i, 1 mM $MgCl_2$ and 50 μM Arsenazo III (pH 7.4). 0.5 mg mitochondrial protein was added to 3 ml medium followed by 6 nmol rotenone, 1 μg oligomycin and either 10 μM Tris-ADP (trace a), no addition (trace b), 10 μM ADP and 4 nmol atractyloside (trace c); or 4 μg bongkrekate (trace d). Further additions as indicated: Tris-succinate (5 mM), $CaCl_2$ (30 nmol), ruthenium red (30 pmol) and EGTA (300 nmol). Temperature 24°C.

The observed effects of the ADP/ATP translocator on Ca^{2+} uptake, retention and set-point could be due to either stimulation of the rate of electrogenic uptake and/or inhibition of efflux. The results of fig.1 clearly indicate stimulation of electrogenic uptake, but the role of efflux inhibition, if any, is not clear. Moreover, the results clearly show that the ADP effect on electrogenic uptake strongly depends on the matrix Ca^{2+} loading because the stimulation of Ca^{2+} uptake is much greater after the second addition of Ca^{2+}. This suggests that the ADP effect is exerted on the matrix face of the inner membrane possibly by increasing Ca^{2+} dissociation from the carrier. To elucidate the contribution from inhibition of Ca^{2+} efflux by ADP, we examined the effect of 3 efflux promotors. Atractyloside (ATR), which inhibits the ADP effect, ruthenium red (RR), which inhibits the electrogenic Ca^{2+} carrier, and NaCl (Na), which promotes Ca^{2+}-Na^+ exchange. Fig.2 shows that ATR induces fast Ca^{2+} efflux only at high Ca^{2+} loading when both ADP and P_i are present. Table 1 shows the results of experiments in which efflux rates from Ca^{2+}-loaded mitochondria (in the presence of ADP and P_i) obtained with RR,

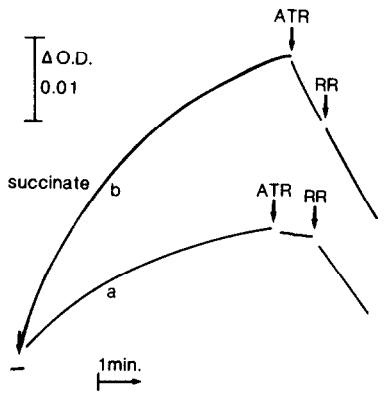

Fig.2. Dependence of atractyloside-induced Ca^{2+} efflux on Ca^{2+} loading. Medium and conditions as in fig.1, except for the omission of Tris-P_i and ADP (trace a). In trace b, 5 mM Tris-P_i and 10 μM ADP were added prior to succinate (5 mM) addition. RR, 30 pmol; atractyloside, 4 nmol.

ATR and Na, alone and in different combinations, were compared. If the ATR effect were due to stimulation of Ca^{2+} efflux alone, we would expect the effect of RR + ATR to be additive. This is clearly not the case, which suggests that ATR inhibits Ca^{2+} uptake. Since ATR alone induced a significantly higher rate of efflux than RR, it appears that in addition to inhibition of the electrogenic carrier, ATR also stimulates Ca^{2+} efflux. The extent of this stimulation can be obtained from the difference between the rate of efflux induced by RR + ATR and RR alone (23.9–18.1 = 5.8). Since the difference between RR + ATR + Na and RR + Na (29.5–24.2 = 5.3) is approximately the same, it appears that Ca^{2+}-Na^+ exchange is not stimulated by ATR. The finding that ATR stimulated the Na^+-independent efflux by about 5.5 nmol/mg protein per min indicates that it inhibited electrogenic transport by approx. 90% (21.4–5.5 = 15.9; 15.9/18.1 = 0.88). Similar calculations verified that these estimates of the effects of ATR are compatible with all the combinations of inhibitors listed in Table 1. If ATR (which inhibits the ADP effect) inhibits 90% of the electrogenic transport at high Ca^{2+} load in the presence of ADP, then ADP appears to stimulate the electrogenic uptake approx. 10-fold. This estimate is compatible with the results of fig.1 (cf. trace a and b after Ca^{2+} addition). The inhibition of Na^+-independent efflux by ADP under the conditions of table 1 is about 25% [of total efflux induced by ATR (21.4), 15.9 is due to inhibition of uptake, thus 5.5/21.4 = 0.26]. The stimulation of electrogenic uptake by ADP is independent of P_i. However, the inhibition of efflux depends on the concentration of P_i (not shown).

Table 1

Effect of atractyloside, ruthenium red and NaCl on Ca^{2+} efflux

Agent	Rate of efflux (nmol/mg protein per min)
RR	18.1
ATR	21.4
NaCl	7.8
RR + ATR	23.9
RR + NaCl	24.2
ATR + NaCl	25.8
RR + ATR + NaCl	29.5

To assess the physiological significance of these results, it is necessary to consider additional factors. Firstly, the set-point depends on the level of Ca^{2+} loading of the matrix. By increasing the concentration of mitochondria, the Ca^{2+} load can be reduced and the set-point can be lowered below 0.1 μM (not shown). Moreover, in the cytoplasm several other factors, such as Mg^{2+}, Na^+ and

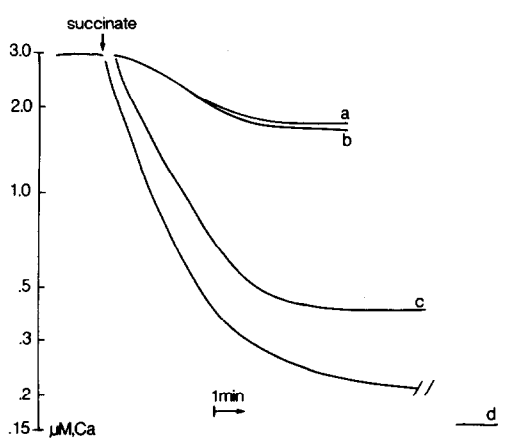

Fig.3. Effect of ADP and spermine on Ca^{2+} set-point. Ca^{2+} transport was followed by a Ca^{2+} electrode. Basic medium (fig.1) was supplemented with 100 mM KCl and 10 mM NaCl; Arsenazo III was omitted. Trace a, without further addition; b, with 1 mM spermine; c, with 0.3 mM MgADP; d, with both ADP and spermine. Ca^{2+} uptake was initiated by the addition of succinate (5 mM).

polyamines, could greatly influence the set-point. These effects on brain mitochondria are shown in fig.3. In a simulated 'physiological' medium containing Mg^{2+}, P_i, KCl, and NaCl, but without ADP and polyamines, Ca^{2+}-loaded mitochondria could only lower external Ca^{2+} to 1.8 μM (curve a). When spermine was added, only a slight effect was observed (curve b). In contrast, when ADP was added, a substantial enhancement of Ca^{2+} accumulation and lowering of the set-point was observed (curve c). When both ADP and spermine were added the set-point could be lowered further to very low levels (curve d). The latter are well within the physiological range. It is interesting to note that in brain mitochondria (unlike liver mitochondria) the effect of polyamines is obtained only when ADP is present.

4. DISCUSSION

The present results demonstrate that brain mitochondria possess the ability to sequester, retain and release Ca^{2+} under physiological conditions provided that the medium is supplemented with adenine nucleotides and polyamines. These metabolites are present in brain cells in concentrations which can control Ca^{2+} transport by mitochondria. Since both spatial and temporal fluctuations in levels of Ca^{2+}, polyamines [10], adenine nucleotides and Na^+ occur in excitable cells, there is reason to believe that mitochondria could play a role in cellular Ca^{2+} regulation. While it is evident that neurotransmitter or hormone-induced Ca^{2+} release does not involve the mitochondria, the reduction of the elevated Ca^{2+} level after excitation may depend, in part, on Ca^{2+} uptake by mitochondria. During prolonged relaxation, Ca^{2+} may exit through the efflux system thus modulating the excitation-relaxation cycle.

The crucial importance of adenine nucleotides for Ca^{2+} sequestration and retention in brain mitochondria is a novel finding of this study. Very recently, it has been shown that in liver mitochondria, locking the adenine nucleotide translocator in the M-state inhibits Ca^{2+} efflux by preventing the Ca^{2+}-induced loss of membrane permeability [10]. In liver, however, the main effect of ADP appears to be on Ca^{2+} efflux, whereas in the brain the main effect is on the uptake by the electrogenic transport carrier.

A model that could explain our observations is as follows: Because the ADP/ATP translocator contains a large number of positive charges [9], its conformation could affect the net surface charge on the M-side of the inner membrane as well as the clustering of negatively charged phospholipids. If, as appears from our results, the uptake rate, at high load of Ca^{2+}, is limited by the rate of Ca^{2+} dissociation from the carrier, the presence of adjacent negatively charged phospholipids could enhance the rate of dissociation. Conversely, the presence of such groups should impede Ca^{2+} association with the Na^+-independent efflux system. In addition, since the membrane surface contributes significantly to Ca^{2+} buffering, a change in surface charge may result in lowering of matrix free Ca^{2+} which should enhance Ca^{2+} uptake and inhibit efflux. This hypothesis is now being tested experimentally.

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