The role of ADP in the modulation of the calcium-efflux pathway in rat brain mitochondria

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(Received 27 April 1984/Accepted 11 September 1984)

The role of ADP in the regulation of Ca^{2+} efflux in rat brain mitochondria was investigated. ADP was shown to inhibit Ruthenium-Red-insensitive H^{+} - and Na^{+} -dependent Ca^{2+} -efflux rates if P_i was present, but had no effect in the absence of P_i . The primary effect of ADP is an inhibition of P_i efflux, and therefore it allows the formation of a matrix Ca^{2+} - P_i complex at concentrations above 0.2 mM-P_i and $25\text{ nmol of Ca}^{2+}/\text{mg of protein}$, which maintains a constant free matrix Ca^{2+} concentration. ADP inhibition of P_i and Ca^{2+} efflux (a) is nucleotide-specific, since in the presence of oligomycin and an inhibitor of adenylate kinase ATP does not substitute for ADP, (b) is dependent on the amount of ADP present, and (c) requires ADP concentrations in excess of the concentrations of translocase binding sites. Brain mitochondria incubated with 0.2 mM-P_i and ADP (a) showed Ca^{2+} -efflux rates dependent on Ca^{2+} loads at Ca^{2+} concentrations below those required for the formation of a P_i - Ca^{2+} complex, and (b) behaved as perfect cytosolic buffers exclusively at high Ca^{2+} loads. The possible role of brain mitochondrial Ca^{2+} in the regulation of the tricarboxylic acid-cycle enzymes and in buffering cytosolic Ca^{2+} is discussed.

Mitochondria have been shown to possess an electrophoretic uniporter for the uptake of Ca^{2+} and a Ruthenium-Red-insensitive pathway for electroneutral Ca^{2+} efflux, which is activated by Na^{+} in brain and heart (Crompton *et al.*, 1976; Nicholls, 1978*a,b*). The existence of these two separate pathways of uptake and release of Ca^{2+} allows a continuous Ca^{2+} cycling that provides the basis for a kinetic regulation of the distribution of Ca^{2+} between cytosol and matrix. At Ca^{2+} concentrations close to those found in the cytosol, Ca^{2+} distribution reflects a steady rather than an equilibrium state (Carafoli, 1979; Nicholls, 1978*b*).

Mitochondria have been assigned two roles in controlling intracellular Ca^{2+} distribution. On the one hand, they could contribute to the maintenance of a low Ca^{2+} concentration in the cytosol (Nicholls & Åkerman, 1982). On the other, they could function in the regulation of the tricarboxylic acid cycle by intramitochondrial Ca^{2+} (Denton *et al.*, 1980; Denton & McCormack, 1980). These

roles are apparently exclusive, since the conditions to be met in order to achieve a Ca^{2+} -buffer role do not coincide with those expected to occur if functioning in the regulation by Ca^{2+} of the tricarboxylic acid cycle.

In addition to the steep dependency of the uniporter on external free $[\text{Ca}^{2+}]$ (Saris & Åkerman, 1980), the Ca^{2+} -buffer role requires both the free mitochondrial Ca^{2+} concentration and Ca^{2+} -efflux rate to be constant and independent of the Ca^{2+} load. Sequestration of cytosolic Ca^{2+} would proceed through the uniporter until the external free Ca^{2+} concentration has fallen to a value at which Ca^{2+} influx balances Ca^{2+} efflux (Nicholls & Åkerman, 1982). However, a regulatory function for mitochondrial Ca^{2+} requires the mitochondrial free Ca^{2+} content to vary with external Ca^{2+} , and, as a consequence, the rate of Ca^{2+} efflux varies with the Ca^{2+} load (Nicholls & Åkerman, 1982).

The rate of Ca^{2+} efflux from mitochondria is known to be affected by the presence of P_i and adenine nucleotides (Tjioe *et al.*, 1970; Lötscher *et al.*, 1980; Roos *et al.*, 1980; Zoccarato & Nicholls, 1981*b*, 1982; Bernardi & Pietrobon, 1982; Sili-prandi *et al.*, 1983; Toninello *et al.*, 1983). Among other effects, P_i has been postulated to activate (Roos *et al.*, 1980) or inhibit (Zoccarato &

Abbreviations used: $\Delta\psi$, mitochondrial membrane potential; ΔpH , pH difference across the mitochondrial inner membrane; pCa^{2+} , the negative logarithm of the free Ca^{2+} concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Nicholls, 1981b) the Ca^{2+} -efflux pathway and to be involved in a Ca^{2+} - P_i symport for Ca^{2+} release (Rugolo *et al.*, 1981) in liver mitochondria. Some of these effects depend on the presence or absence of adenine nucleotides in the incubation medium and on the magnitude of the membrane potential (Zoccarato & Nicholls, 1982). Heffron & Harris (1981) have reported that ADP inhibits the H^+ -dependent Ca^{2+} -efflux pathway and that it has no effects on the Na^+ -dependent efflux in rat heart mitochondria.

Whereas the role of P_i has been convincingly explained in terms of a P_i - Ca^{2+} complex (Weinbach & Von Brand, 1965) and the conditions for the formation of such a complex have been elucidated (Zoccarato & Nicholls, 1982), the role of adenine nucleotides is not clearly established. Since the dependency of the Ca^{2+} -efflux rate on the Ca^{2+} load is critical in determining the role of mitochondrial Ca^{2+} , we have investigated the effects of phosphate and ADP on the H^+ - and Na^+ -dependent Ca^{2+} -efflux pathways of rat brain mitochondria and on the ability of mitochondria to buffer external Ca^{2+} .

Materials and methods

Male Wistar rats fed *ad libitum* on a standard laboratory diet were used throughout.

Isolation of non-synaptic and synaptic mitochondria

The synaptosomal and non-synaptosomal brain mitochondrial fractions were prepared by the method of Nicholls (1978a), with the following modifications. The brain homogenate [1:10 (w/v) in 0.32M-sucrose/10mM-Tris/HCl/1mM-EDTA/0.1% bovine serum albumin (fatty acid-free), pH 7.4] was centrifuged at 2000g for 3 min. The supernatant was further centrifuged at 12500g for 10 min, the resulting pellet being the crude mitochondrial fraction. This was then resuspended in 'low-osmolarity medium' [0.12M-mannitol/0.03M-sucrose/10mM-Tris/HCl/0.025mM-EDTA (pH 7.4)/3% Ficoll], incubated for 30 min at 4°C (Lai & Clark, 1976), layered over a discontinuous Ficoll gradient, consisting of two layers of 7.5% or 13% (w/v) Ficoll in 0.32M-sucrose/1mM-EDTA/10mM-Tris/HCl (pH 7.4)/0.1% bovine serum albumin (fatty acid-free), and centrifuged at 98000g for 30 min. The pellet was resuspended (15–20 mg of protein/ml) in 0.32M-mannitol/10mM-Tris/HCl/0.1% bovine serum albumin (fatty acid-free), pH 7.4.

Measurement of the efflux of Ca^{2+} from brain mitochondria

This was done by using the metallochromic indicator Arsenazo III (Scarpa, 1979) and an

Aminco DW2a dual-wavelength spectrophotometer, at the wavelength pair 675–685 nm. Mitochondria (2 mg of protein) were added to 2.5 ml of 'incubation medium' [0.32M-mannitol/10mM-Tris/HCl/20mM-KCl/0.1% bovine serum albumin (fatty acid-free), pH 7.4], 0.2mM-Arsenazo III (purified by the method of Scarpa, 1979), 2.5mM-succinate (K^+ salt) and 2.4 μM -rotenone, and different additions as indicated in the Figures. A calibrated addition of CaCl_2 was made to provide the desired loading. After Ca^{2+} additions, the uptake was nearly complete in about 2–3 min. The active influx was stopped by adding Ruthenium Red (1.2 μM) and the initial rate of absorbance change was recorded. The amount of intramitochondrial Ca^{2+} (Ca^{2+} available for release) was estimated from the total absorbance change produced by the addition of 10mM-NaCl and 1.2 μM -FCCP or 0.2 μM -ionophore A23187.

Measurement of the $p\text{Ca}^{2+}$ of the extramitochondrial medium

This was done with an ion-selective electrode (Ronner, 1979) with a pH-reference electrode immersed in a thermostatically controlled chamber of 1.3 ml volume. To calibrate the electrode assembly, and to buffer extramitochondrial free Ca^{2+} , Ca^{2+} /nitrilotriacetate buffers were used (apparent stability constant, at 25°C and pH 7.4, $2.81 \times 10^4 \text{ M}^{-1}$). Routinely, the electrode was calibrated by adding to 'incubation medium', containing 2.5mM-succinate (K^+ salt), 2.4 μM -rotenone and 2mM-potassium nitrilotriacetate, 5.7 μM -, 18.44 μM - or 55.6 μM - CaCl_2 , giving (at 25°C and pH 7.4) free Ca^{2+} activities of 0.1 μM ($p\text{Ca}^{2+}$ 7.0), 0.32 μM ($p\text{Ca}^{2+}$ 6.5) and 1 μM ($p\text{Ca}^{2+}$ 6.0) respectively. Further additions to the incubations were made as indicated in the legends to Figures. The protein content per assay was 0.8–1 mg.

Membrane potential

The mitochondrial membrane potential ($\Delta\psi$) was measured by monitoring the movements of tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a tetraphenylphosphonium-selective electrode prepared by the method of Kamo *et al.* (1979), with a pH electrode as the reference electrode.

Mitochondrial incubations were performed as described for Ca^{2+} movements (except that nitrilotriacetate was omitted), in the presence of 1.25 μM -tetraphenylphosphonium.

Mitochondrial volume measurements

Mitochondrial volume was determined from the distribution of $^3\text{H}_2\text{O}$ (0.5 $\mu\text{Ci/ml}$), by using [$\text{U}-^{14}\text{C}$]sucrose (0.03 $\mu\text{Ci/ml}$) as the extramatrix marker.

P_i fluxes

To determine P_i fluxes, mitochondria were incubated at 25°C in 'incubation medium' with 2mg of mitochondrial protein/ml, 2.5mM-succinate (K^+ salt) and $2.4\mu\text{M}$ -rotenone (for further additions see Figure legends). At intervals, 0.5ml samples of the incubation were transferred into centrifuge tubes containing ice-cold *N*-ethylmaleimide (100nmol/mg of protein) and centrifuged for 1.5min in an Eppendorf 5414 table centrifuge. The pellet was rinsed with ice-cold 'incubation medium' and extracted with 10% trichloroacetic acid. P_i content was determined by the method of Lanzetta *et al.* (1979).

ADP uptake

ADP uptake in brain mitochondria was measured by the inhibitor-stop procedure. Mitochondria (2mg of protein/ml) were incubated at 25°C in 'incubation medium' containing 2.5mM-succinate (K^+ salt), 0.6mM- P_i , 0.1–0.4mM-[$\text{U}-^{14}\text{C}$]-ADP (0.05 $\mu\text{Ci}/\text{ml}$) and $4\mu\text{M}$ -oligomycin in the presence or absence of 20 μM -atractyloside. After incubation for 5min, 2.5mM-carboxyatractyloside was added, and samples were centrifuged for 1.5min in an Eppendorf centrifuge. The supernatant was discarded and the pellet was rinsed in 100 μl of 10% (w/v) sodium dodecyl sulphate. The radioactivity of the pellet was measured in a liquid-scintillation spectrometer. In all experiments the sucrose-impermeable space was determined by using [$\text{U}-^{14}\text{C}$]sucrose in place of substrate.

Determination of mitochondrial disruption

These experiments were conducted to determine whether mitochondria become leaky to low- M_r anions during P_i -efflux experiments. For this

purpose, the latency of the matrix enzymes fumarase and glutamate dehydrogenase was determined at different times during the incubation. Glutamate dehydrogenase and fumarase activities were assayed as described by Kaplan & Pedersen (1983), and protein was measured by the biuret method. For both assays, Lubrol activation was accomplished by incubating mitochondria with 1mg of Lubrol/mg of mitochondrial protein for at least 15min at 0°C . The mean latency indexes (i.e. specific activity in the presence of Lubrol/specific activity in the absence of Lubrol) of glutamate dehydrogenase and fumarase were 65 and 4 respectively and did not change with increasing incubation times.

Results

Interaction of phosphate and ADP with Ca^{2+} efflux

The addition of P_i induces Ca^{2+} efflux in rat liver mitochondria incubated in the absence of ATP (Siliprandi *et al.*, 1979), and Ca^{2+} efflux is directly dependent on internal P_i concentrations (Roos *et al.*, 1980). This situation is reversed in the presence of ATP, and under these conditions Ca^{2+} efflux is higher with lower P_i concentrations (Zoccarato & Nicholls, 1982). We have found that the Ca^{2+} efflux rate shows a similar P_i -dependency in rat brain mitochondria. Fig. 1 shows that the H^+ - and Na^+ -dependent rates of Ca^{2+} release at a constant Ca^{2+} load increase with increasing [P_i] in the absence of ADP, and decrease with increasing [P_i] in the presence of ADP. The rates of Ca^{2+} efflux follow opposite trends, depending on the presence or absence of ADP, and converge to a same basal rate at zero P_i . This suggests that ADP does not affect the Ca^{2+} -release rate in the absence

Fig. 1. Effect of phosphate and ADP on Ruthenium-Red-induced Ca^{2+} efflux, in the presence (a) or absence (b) of 10mM- Na^+ , in rat brain mitochondria

Mitochondria (2mg of protein) were incubated with potassium succinate and a fixed Ca^{2+} load (25 nmol/mg of protein) and various amounts of P_i , in the presence ($\square$, $\blacksquare$) or absence ($\triangle$, $\blacktriangle$) of ADP (200 μM); 0.4nmol of oligomycin/mg of protein and $2.4\mu\text{M}$ -rotenone were present in both experiments. Ruthenium Red was added when the uptake of Ca^{2+} was ended.

of P_i or that ADP effects are P_i -dependent. The small effect of this nucleotide on the H^+ - and Na^+ -dependent Ca^{2+} -efflux rates in mitochondria incubated in the absence of P_i (Fig. 2a) could correspond to the presence of endogenous P_i in our brain mitochondria preparations (4 nmol/mg of protein).

When brain mitochondria are incubated in the presence of ADP, oligomycin and P_i , both the H^+ -

and Na^+ -dependent Ca^{2+} -efflux rates follow a similar dependency on Ca^{2+} loads (results not shown) or Ca^{2+} available for release (Fig. 2c). At low matrix $[Ca^{2+}]$, the rate of Ca^{2+} release depends on the Ca^{2+} load, which, in turn, is fixed by external $[Ca^{2+}]$. At Ca^{2+} loads higher than 30 nmol/mg, the Ca^{2+} -efflux rate becomes constant, indicating that intramitochondrial Ca^{2+} is buffered by the presence of the internal P_i - Ca^{2+} complex (Zoccarato & Nicholls, 1982).

Inhibition by ADP of phosphate efflux from rat brain mitochondria

The results reported above indicate that P_i is involved in ADP effects. ADP could stabilize P_i within mitochondria, and thus modify the matrix Ca^{2+} -buffering power and the Ca^{2+} -efflux rate. We have investigated this possibility by studying the effect of ADP on phosphate efflux.

The results in Figs. 3 and 4 show that, in the absence of ADP, P_i and Ca^{2+} movements are essentially parallel. In agreement with the results of Zoccarato *et al.* (1981) and Wehrle & Pedersen (1979), both uptake and efflux of P_i from mitochondria increase in the presence of Ca^{2+} . Moreover, in the presence of excess EDTA, P_i release is totally blocked, and uptake is smaller (Fig. 3a). P_i efflux is higher in the presence of added Ca^{2+} , especially when Ca^{2+} release is activated with Na^+ , and is severely decreased when Ca^{2+} uptake is inhibited (see Ruthenium Red effects; Fig. 3b). However, in the presence of ADP, the amounts of P_i and Ca^{2+} taken up by mitochondria are high, whereas P_i efflux is hardly detectable. In addition, the gradual depolarization occurring in mitochondria incubated with P_i and Ca^{2+} is reversed (Fig. 4).

Since there was no mitochondrial disruption during P_i -efflux experiments (see the Materials and methods section) and the mitochondrial membrane potential measured in parallel experiments was at all times above 185 mV (Fig. 4), the observed efflux rates of P_i do not result from mitochondrial damage.

Effect of ADP on the matrix free Ca^{2+} -buffering power

The results shown above indicated that ADP was able to inhibit Ca^{2+} -dependent- P_i efflux and P_i -dependent Ca^{2+} release in rat brain mitochondria. The stabilization of P_i within mitochondria brought about by ADP could modify the intramitochondrial Ca^{2+} -buffering power. This would then result in a parallel modification of the rate of Ca^{2+} efflux. The dependency of the matrix free Ca^{2+} -buffering power with respect to ADP concentration was assayed in the following experiment. Mitochondria were given a fixed Ca^{2+} and

Fig. 2. Effect of ADP and P_i on the rate of Ca^{2+} efflux in the presence or absence of Na^+

Mitochondria (2 mg of protein) were incubated with succinate (K^+ salt), 0.4 nmol of oligomycin/mg of protein (except in a) 2.4 μM -rotenone and increasing amounts of Ca^{2+} , and Ca^{2+} efflux was initiated by Ruthenium Red addition. (a) Ruthenium-Red-insensitive Ca^{2+} efflux in the absence ($\circ$) or presence ($\blacktriangle$) of 50 μM -ADP. (b) Na^+ -activated Ca^{2+} efflux in the absence ($\square$) or presence ($\blacksquare$) of 200 μM -ADP. (c) Ca^{2+} efflux in the presence of 200 μM - P_i and 200 μM -ADP and in the absence ($\triangle$) or presence ($\circ$) of 10 mM- $NaCl$. Note the different scales used.

Fig. 3. Effect of Ca^{2+} and ADP on phosphate-efflux rate in rat brain mitochondria

Mitochondria were incubated as described in the Materials and methods section in the presence of 0.4 nmol of oligomycin/mg of protein, 2.4 μM -rotenone, 0.14 mM- P_i and 0.14 mM-ADP (●), 0.14 mM-ADP + 25 nmol of Ca^{2+} /mg of protein (□), 0.1 mM-EDTA (▲), 25 nmol of Ca^{2+} /mg of protein (■), 25 nmol of Ca^{2+} /mg of protein + 10 mM- NaCl (◆), 25 nmol of Ca^{2+} /mg of protein + 12 μM -Ruthenium Red (○), or with no additions (△). After the times indicated, samples were withdrawn and assayed for phosphate. The average P_i -efflux rates observed in the interval between 3 and 15 min after P_i addition were 0.54 ± 0.04 (△), 0.54 ± 0.10 (○), 0.77 ± 0.09 (■) and 0.87 ± 0.13 (◆), nmol of P_i /min per mg of protein. The significance of the differences in P_i -efflux rates between incubations with P_i (△) or P_i + Ca^{2+} + Ruthenium Red (○) and P_i + Ca^{2+} (■) or P_i + Ca^{2+} + Na^+ (◆) was $P < 0.025$. *† The significance of the difference in P_i contents at times 0–3 min between incubations with P_i + Ca^{2+} (■) and P_i (△) (*), and between incubations with P_i + Ca^{2+} (■) and P_i + Ca^{2+} + Na^+ (◆) or P_i + Ca^{2+} + Ruthenium Red (○) (†), evaluated by Student's t test, was $P < 0.0005$ for (*) and $P < 0.0025$ for (†).

Fig. 4. Effects of phosphate, ADP and Na^+ on Ca^{2+} and tetraphenylphosphonium distribution

(a) Mitochondria (0.8 mg of protein) were incubated with succinate (K^+ salt), 0.4 nmol of oligomycin/mg of protein and 2.4 μM -rotenone. In trace a, 0.14 mM-ADP and 0.14 mM- P_i were initially present; in trace b, no additions; in trace c, 0.14 mM- P_i was initially present; and in trace d, 0.14 mM- P_i was initially present and 10 mM- NaCl (Na^+) was added as indicated. When added, ADP concentration was 0.14 mM. (b) Mitochondria were incubated as in (a), in the presence of 1.25 μM -tetraphenylphosphonium and 10 nmol of Ca^{2+} /mg of protein (see deflection at the point of Ca^{2+} addition), with the following additions: —, 12 μM -Ruthenium Red (RR); ----, 0.14 mM-ADP (ADP); - - - - - , 10 mM- NaCl (Na^+) and 0.14 mM-ADP (ADP).

P_i load in the presence of variable amounts of ADP. Total Ca^{2+} -release rate was then determined after the additions of Ruthenium Red, Na^+ (10 mM) and FCCP (1.2 μM). We have confirmed

the results of Goldstone *et al.* (1983) showing that the magnitude of the Ca^{2+} load limits the Ca^{2+} -release rate measured after the simultaneous addition of Ruthenium Red, Na^+ and 0.2 μM -

Fig. 5. Effect of the presence of various amounts of ADP on the rate of Ca^{2+} efflux

Rat brain mitochondria were incubated with 25 nmol of Ca^{2+} /mg of protein, 0.2 mM- P_i , 0.4 nmol of oligomycin/mg of protein and 2.4 μM -rotenone. (a) Ca^{2+} efflux was estimated after the addition of Ruthenium Red (12 μM) in the presence ($\circ$) or absence (Δ) of 10 mM-NaCl. Note the difference in scales between Ca^{2+} -efflux rates in the presence ($\circ$) or absence (Δ) of NaCl. (b) The Ca^{2+} -efflux rate was estimated after the addition of Ruthenium Red (12 μM), 10 mM-NaCl and 1.2 μM -FCCP.

A23187 or 1.2 μM -FCCP (results not shown). Therefore any change in the mitochondrial buffering power by ADP should be manifested as a decreased amount of Ca^{2+} released. Fig. 5(b) shows that the rate of Ca^{2+} release measured under these conditions decreased with the amount of ADP added, and, in the range of ADP concentrations used, did not show saturation. In addition, the H^+ - and Na^+ -dependent Ca^{2+} -efflux rates (Fig. 5a) also decrease with increasing ADP concentrations. These results indicate that the Ca^{2+} -buffering power of mitochondria increases with [ADP]. Since it was previously shown that ADP effects depended on $[\text{P}_i]$, these results suggest that inhibition of P_i efflux by ADP depends on ADP concentration.

Steady-state regulation of extramitochondrial $[\text{Ca}^{2+}]$

Rat brain mitochondria incubated in a Ca^{2+} -buffered medium in the presence of P_i and ADP and free Ca^{2+} concentrations lower than 0.23 μM are unable to maintain a constant external Ca^{2+} concentration (Fig. 6a). A perfect buffering effect is only achieved at high Ca^{2+} loads (Fig. 6a). Those

two different responses of brain mitochondria to regulate extramitochondrial Ca^{2+} resemble the dual behaviour of the Ca^{2+} -efflux rate at low and high Ca^{2+} loads shown in Fig. 2. This could be predicted if the steady-state Ca^{2+} distribution reflects a kinetic rather than a thermodynamic equilibrium. It should be noted that the total variations of extramitochondrial $[\text{Ca}^{2+}]$ in mitochondria incubated with constant $[\text{P}_i]$ and $[\text{ADP}]$ are small and within the range of concentrations of free Ca^{2+} in the cytosol of most cells in the resting state (Murphy *et al.*, 1980; Marban *et al.*, 1980).

Fig. 6(b) shows that the steady-state Ca^{2+} distribution is dependent on the amount of ADP present in the incubation medium, higher ADP concentrations resulting in lower external $[\text{Ca}^{2+}]$. Moreover, in agreement with Zoccarato *et al.* (1981), we have found that ATP is unable to substitute for ADP: in the presence of oligomycin and P^1P^5 -bis-(5'-adenosyl) pentaphosphate, an inhibitor of adenylate kinase, the steady-state Ca^{2+} distribution attained after ATP addition is set at much higher concentrations of external Ca^{2+} . This takes place after a substantial lag, which possibly reflects leaky ADP formation. After a re-setting of Ca^{2+} steady-state distribution by Na^+ addition, ATP-incubated mitochondria undergo a drastic fall in Ca^{2+} content, contrary to the results obtained in the presence of ADP (Fig. 6b).

Discussion

ADP and phosphate effects

It has long been known that P_i is able to induce a spontaneous Ca^{2+} efflux in Ca^{2+} -loaded mitochondria (Lehninger *et al.*, 1967). Several reports also state that efflux of P_i and adenine nucleotides accompanies Ca^{2+} release, and that the mitochondrial membrane potential is dissipated during the process (Nicholls & Scott, 1980; Zoccarato & Nicholls, 1981b, 1982; Rugolo *et al.*, 1981; Zoccarato *et al.*, 1981). Nicholls & Scott (1980) and Zoccarato & Nicholls (1982) have found that liver or brain mitochondria can sustain high Ca^{2+} and P_i loads when incubated with adenine nucleotides. They have shown that, under these conditions: (a) losses of adenine nucleotides, Ca^{2+} and P_i were prevented; (b) $\Delta\psi$ and ΔpH were maintained; and (c) the effect of P_i on Ca^{2+} efflux consisted of a substantial decrease by small increases of P_i , followed by attainment of a constant efflux rate at P_i concentrations higher than 1 mM. Zoccarato *et al.* (1981) studied the nucleotide-specificity of the process and found that, in the presence of inhibitors of ATP synthetase and adenylate kinase, only ADP was effective in retarding efflux of Ca^{2+} and P_i . Meisner & Klingenberg (1968) also concluded that the decrease in adenine nucleotide

Fig. 6. Steady-state Ca^{2+} distribution in rat brain mitochondria incubated in the presence of P_i and ADP (a) and after replacement of ADP by ATP (b)

(a) Mitochondria (0.6 mg of protein) were incubated with 0.8 mM- P_i , 0.2 mM-ADP, 2.4 μM -rotenone and 0.4 nmol of oligomycin/mg of protein in a nitrilotriacetate/ Ca^{2+} buffer in the presence of succinate (K^{+} salt). The steady-state pCa^{2+} after the addition of different Ca^{2+} loads was estimated with a Ca^{2+} -sensitive electrode. (b) Mitochondria (0.8 mg of protein) were incubated with 1 mM- P_i , 2.4 μM -rotenone, 0.4 nmol of oligomycin/mg of protein, 45 μM - P^{5-} -bis-(5'-adenosyl) pentaphosphate in a nitrilotriacetate/ Ca^{2+} buffer in the presence of succinate (K^{+} salt) and 37.16 μM - Ca^{2+} (Ca^{2+}). At the arrow, 50 μM -ADP (trace a) or 50 μM -ATP (trace b) was added. Subsequent additions were Na^{+} (10 mM- NaCl) and (1) 50 μM - or (2) 100 μM -ADP.

efflux was brought about exclusively by ADP. We have confirmed most of these findings, including nucleotide specificity (Fig. 6b), and extended them to the Na^{+} -dependent efflux pathway.

Brain mitochondria incubated with small amounts of P_i and Ca^{2+} in the absence of ADP showed a gradual dissipation of $\Delta\psi$ that was accompanied by a parallel efflux of P_i and Ca^{2+} (Figs. 3 and 4). Since the process took place at high $\Delta\psi$, it is unlikely that Ca^{2+} efflux occurs through a reversal of the uniporter, except if the depolarization is due to membrane damage (Nicholls & Åkerman, 1982) and an increasing number of mitochondria being destroyed (Hunter *et al.*, 1976). However, this interpretation does not explain the excellent reversibility of the process by ADP (Fig. 4).

In the presence of ADP, P_i efflux is inhibited and, provided that enough P_i and Ca^{2+} are present, a stable Ca^{2+} - P_i complex is formed in the mitochondrial matrix. As pointed out by Zoccarato & Nicholls (1982), the Ca^{2+} - P_i complex

maintains a constant free matrix Ca^{2+} concentration; consequently, the activities of the Ca^{2+} -efflux pathways, which depend on internal Ca^{2+} concentration, become constant. At a Ca^{2+} concentration of 25 nmol/mg of protein, this complex is formed at P_i concentrations corresponding to 0.2 mM external P_i (Fig. 1); accordingly, when $[\text{P}_i]$ is fixed at 0.2 mM, the formation of the complex requires 25 nmol of Ca^{2+} /mg of protein (Fig. 2).

The finding that ADP inhibits the Na^{+} -dependent Ca^{2+} efflux through the formation of a P_i - Ca^{2+} complex (Figs. 1 and 2) contradicts previous results from Harris *et al.* (1979) and Heffron & Harris (1981), who reported that ADP was unable to inhibit the Na^{+} -activated Ca^{2+} efflux. This discrepancy probably arises from the fact that those authors used very low ADP concentrations and, as shown in Fig. 5, ADP effects are concentration-dependent.

The stabilization of P_i -incubated mitochondria by ATP or ADP involves the adenine nucleotide translocase, as manifested by the fact that bong-

kreic acid produces the same effect (Harris, 1979), and ATP is no longer effective in the presence of carboxyatractylate (Nicholls & Scott, 1980). The binding of ADP to translocase would give rise to the block in adenine nucleotide efflux reported by Meisner & Klingenberg (1968) and would provide mitochondria with the permeability properties that allow the maintenance of stable membrane potentials (Harris, 1979).

Two lines of evidence suggest that ADP requirements are not exclusively due to the binding of ADP to translocase. (a) The maximal inhibitory effect of ADP is attained at concentrations higher than 0.3 mM (Fig. 5). These values are much higher than those required to saturate the back-exchange reaction and the ADP-translocase-binding sites facing the extramitochondrial medium in liver mitochondria (Nohl & Klingenberg, 1978). (b) At 0.1 mM-ADP the effect of ADP on Ca^{2+} efflux is still below saturation (Fig. 5), even though the amount of ADP taken up by brain mitochondria is close to 2 nmol/mg of protein, which is more than the maximum number of translocase binding sites within mitochondria [0.2 $\mu\text{mol/g}$ of protein in liver (Klingenberg *et al.*, 1971; Vignais *et al.*, 1973) and 1.6 $\mu\text{mol/g}$ of protein in heart (Klingenberg *et al.*, 1975)].

Physiological aspects

Our results show that brain mitochondria incubated with 0.8 mM- P_i and 0.2 mM-ADP are able to maintain a free Ca^{2+} concentration in the external medium that varies slightly between 0.16 and 0.25 μM , within the range of cytosolic $[\text{Ca}^{2+}]$ (Murphy *et al.*, 1980; Marban *et al.*, 1980). At the same time the internal Ca^{2+} load varies from no added Ca^{2+} to 150 nmol/mg of protein (Fig. 6), indicating a function in cytosolic Ca^{2+} buffering (Nicholls & Scott, 1980). Since the concentration of free matrix Ca^{2+} increases after the uptake of increasing Ca^{2+} loads, it follows that increasing Ca^{2+} concentrations would activate the tricarboxylic acid-cycle enzymes. Then, provided that the cytosol has a P_i -buffering capacity similar to that of the Ca^{2+} buffer used (for a review, see Williamson *et al.*, 1981), at these ADP and P_i concentrations brain mitochondria could perform both functions simultaneously, a possibility pointed out by Zoccarato & Nicholls (1981a).

In the brain, increases in cytosolic Ca^{2+} concentration are associated with depolarization of the nerve endings and neurotransmitter release. These events are accompanied by ATP hydrolysis and increases in ADP and P_i concentrations (Scott & Nicholls, 1980). A rapid sequestration of cytosolic Ca^{2+} in mitochondria would help recovery of the nerve terminals (Åkerman & Nicholls, 1983) and,

according to our results, this would be facilitated by the simultaneous increase of ADP and P_i .

The physiological relevance of a simultaneous uptake of ADP and Ca^{2+} in the activation of the tricarboxylic acid cycle during State-3 respiration has been pointed out by Bernardi & Azzone (1983). The exact requirements for ADP and Ca^{2+} of the brain Ca^{2+} -sensitive tricarboxylic acid-cycle enzymes are unknown, but in liver and heart mitochondria the activities of NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase are regulated by Ca^{2+} and ADP through modifications in the K_m values for isocitrate or oxoglutarate (McCormack & Denton, 1979; Aogaichi *et al.*, 1980). The findings of the present study suggest that, in the resting state, the extremely low ADP concentrations found in brain cytosol (20 μM) in ^{31}P -n.m.r. studies *in vivo* (Prichard *et al.*, 1983) do not allow an efficient buffering of free matrix Ca^{2+} (Figs. 5 and 6), and the mitochondrial Ca^{2+} content would be necessarily low. However, during depolarization the simultaneous uptake of ADP and Ca^{2+} could allow a transient increase in matrix Ca^{2+} concentration that could help the activation of the tricarboxylic acid-cycle enzymes and the rapid rebuilding of the high-energy stores.

This work was supported by a grant from the Caja de Ahorros y Monte de Piedad to J. V. and by grants from the Fondo de Investigaciones Sanitarias and Comisión Asesora de Investigación Científica y Técnica. We thank Miss Maria Victoria Mora Gil for her technical assistance.

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