The Nature of the Calcium Ion Efflux Induced in Rat Liver Mitochondria by the Oxidation of Endogenous Nicotinamide Nucleotides

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Ca^{2+} efflux from rat liver mitochondria can occur when endogenous nicotinamide nucleotides are oxidized. It is suggested that nicotinamide nucleotide oxidation induced by acetoacetate sensitizes the mitochondria to damage resulting from the accumulation of Ca^{2+} in the presence of P_i . Thus, acetoacetate-induced Ca^{2+} efflux is associated with a loss of respiratory control. Both the effluxes induced by acetoacetate and by high Ca^{2+} accumulation are prevented by ATP plus oligomycin, although these agents do not prevent the endogenous nicotinamide nucleotides from becoming oxidized on addition of acetoacetate. Acetoacetate addition only results in Ca^{2+} release if the Ca^{2+} and P_i concentrations are above a critical value. The acetoacetate-induced Ca^{2+} efflux is exactly paralleled by the virtually complete collapse of the membrane potential. The presence of acetoacetate decreases the concentration of total Ca^{2+} necessary to induce mitochondrial damage by about 130 nmol of Ca^{2+} /mg of protein. It is concluded that acetoacetate-induced efflux occurs by reversal of the Ca^{2+} uniporter after the collapse of the membrane potential.

Mitochondria from both excitable and non-excitable tissues possess independent pathways for Ca^{2+} uptake and release (Crompton *et al.*, 1976, 1978; Puskin *et al.*, 1976; Azzone *et al.*, 1977; Crompton & Heid, 1978; Åkerman, 1978; Nicholls, 1978*a,b*; Nicholls & Scott, 1979, 1980). The activity of the efflux pathway is Na^+ -independent in the case of liver mitochondria (Puskin *et al.*, 1976; Crompton *et al.*, 1978; Nicholls, 1978*b*; Åkerman, 1978) and Na^+ -dependent in the case of heart or brain mitochondria (Crompton *et al.*, 1976, 1978; Crompton & Heid, 1978; Nicholls, 1978*a*; Nicholls & Scott, 1979*a,b*). The efflux pathway is unambiguously identifiable by its ability to catalyse efflux even when $\Delta\psi$ is sufficiently high to preclude Ca^{2+} loss from the matrix by reversal of the Ca^{2+} uniporter (Puskin *et al.*, 1976; Nicholls, 1978*a,b*; Nicholls & Scott, 1980).

In contrast with this finely regulated efflux mechanism, a number of incubation conditions *in vitro* can induce gross Ca^{2+} loss from the matrix as a

consequence of the decrease in $\Delta\psi$ owing to damage to the mitochondrial integrity. This efflux is characterized by its time-dependency (for review see Bygrave, 1978), its requirement for a threshold concentration of Ca^{2+} or P_i (Rossi & Lehninger, 1964), by a decreased light-scattering (Chappell & Crofts, 1965), by a loss of inner-membrane permeability barriers (Gazzotti, 1975), by a net loss of matrix adenine nucleotides (Ernster, 1956; Meisner & Klingenberg, 1968; Out *et al.*, 1971; Sul *et al.*, 1976; Prpić *et al.*, 1978; Nicholls & Scott, 1980), and by a sensitization by agents such as atractylate (Asimakis & Sordahl, 1977; Nicholls & Scott, 1980) and phosphoenolpyruvate (Chudapongse & Haugaard, 1973; Peng *et al.*, 1974; Sul *et al.*, 1976; Roos *et al.*, 1978) and is antagonized by ATP, ADP or oligomycin alone or in combination (Rossi & Lehninger, 1964; Lehninger *et al.*, 1967; Bygrave, 1978). It is significant that mitochondria from tumour cells (McIntyre & Bygrave, 1974) or from the livers of rats that have been pretreated with glucagon (Prpić *et al.*, 1978) are unusually resistant to this Ca^{2+} -induced damage.

Recently it has been reported that increased oxidation of matrix nicotinamide nucleotides induces a net efflux of Ca^{2+} from rat liver

Abbreviations used: pCa_{50} , the negative logarithm of the free Ca^{2+} concentration in the extramitochondrial compartment; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane sulphonic acid; $\Delta\psi$, membrane potential.

mitochondria (Lehninger *et al.*, 1978; Fiskum & Lehninger, 1979). In view of the regulatory consequences of such a mechanism, we have investigated this efflux to ascertain the category to which it should be assigned. We conclude that nicotinamide nucleotide oxidation greatly sensitizes the mitochondria to damage during Ca^{2+} uptake, that release occurs as a consequence of a collapse in $\Delta\psi$ and that, although this interesting observation may well throw light on the factors that stabilize Ca^{2+} within the mitochondrial matrix, it is unlikely to represent a physiologically relevant mechanism for the regulation of cytosolic free Ca^{2+} concentrations.

Experimental

Materials

[^3H]Methyltriphenylphosphonium bromide was obtained from New England Nuclear Chemicals G.m.b.H., Freiech, Germany. $^{45}\text{CaCl}_2$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Mitochondria

Mitochondria were prepared from the livers of female Wistar strain rats, as previously described (Heaton & Nicholls, 1976). The mitochondria were stored at 0°C in 250 mM-sucrose/5 mM-Tes (sodium salt) (pH 7.4) for not more than 4 h before use. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Methods

pCa_0^{2+} was determined directly in mitochondrial incubations by means of a Ca^{2+} -selective electrode (Radiometer type F2112Ca) with a KCl reference electrode (Radiometer type K801) as previously described (Nicholls, 1978b). The volume of the incubation chamber was 0.35 ml. The initial Ca^{2+} content of media was determined by a Corning-Eel model 240 mark II atomic-absorption spectrophotometer, and allowance for this was made in all calculations.

Respiration was determined with a Clark-type oxygen electrode in a chamber with a volume of 0.35 ml. $\Delta\psi$ and $^{45}\text{Ca}^{2+}$ accumulation were determined simultaneously by incubating mitochondria (1.5 mg of protein/ml of incubation) at 30°C and pH 7.0 in a medium containing 75 mM-NaCl, 10 mM-Tes (sodium salt), 32 μM -albumin (bovine, fraction V), 2 mM-succinate (sodium salt), 1 μM -rotenone, 2 mM-nitrotriacetate (sodium salt), 2 mM- P_i (sodium salt), 5 μM -tetraphenylboron (sodium salt), 1 μM -[^3H]methyltriphenylphosphonium bromide (0.4 $\mu\text{Ci}/\text{ml}$) and various concentrations of $^{45}\text{Ca}^{2+}$ (0.05 $\mu\text{Ci}/$

ml). Methyltriphenylphosphonium is a lipophilic cation, which has been shown to distribute, in the presence of tetraphenylboron, according to $\Delta\psi$ across the inner membrane of a variety of mitochondria (Bakeeva *et al.*, 1970; Scott & Nicholls, 1980). Albumin was present in order to eliminate any contributory effects of fatty acids or lysophospholipids towards mitochondrial damage. At defined times, 200 μl portions of the incubation were filtered through 0.6 μm pore-size cellulose acetate filters. The accumulation of [^3H]methyltriphenylphosphonium bromide and $^{45}\text{Ca}^{2+}$ within the mitochondria was calculated, after allowing for the volume of extramitochondrial fluid entrapped on the filter. As no isotopic marker for the extramitochondrial space was included in the incubation, this parameter was estimated from the volume of incubation medium required to form a spot of equivalent size on control filters. As only 5% of the activities of ^3H and $^{45}\text{Ca}^{2+}$ calculated on this basis was extramitochondrial under control conditions, this approximation was judged to be satisfactory. $\Delta\psi$ was calculated assuming a constant matrix volume of 0.7 $\mu\text{l}/\text{mg}$ of protein. This further approximation will result in an over-estimate of $\Delta\psi$ values when the mitochondrial matrix is swollen.

The fluorescence attributable to nicotinamide nucleotides within the mitochondrial matrix was monitored with an Eppendorf model 1101M photometer with fluorescence attachment and 313–366 nm primary filter and a secondary filter transmitting above 430 nm.

Results and Discussion

When mitochondria are added to a medium containing 2 mM- P_i and 54 nmol of Ca^{2+}/mg of protein (Fig. 1a), Ca^{2+} is accumulated until pCa_0^{2+} increases to 6.2, after which a slow oscillation occurs until the pCa_0^{2+} finally regains a value of 6.2. During this oscillation the respiratory rate steadily increases, suggesting that the mitochondria are experiencing the early stages of damage to their integrity. It is notable that the increased respiration persists even when pCa_0^{2+} has returned to a value of 6.2. As the activity of the Ca^{2+} uniport is limited at this pCa_0^{2+} to about 5 nmol of $\text{Ca}^{2+}/\text{min}$ per mg of protein (Hutson, 1977; Nicholls, 1978b), it is apparent that the increased respiration cannot be ascribed to an increased Ca^{2+} cycling. The mitochondria retain a slight respiratory control after 5 min (Fig. 1a).

The presence of ATP and oligomycin in the initial incubation (Fig. 1b) abolishes the oscillation in pCa_0^{2+} , prevents the steady increase in respiration and allows a considerably higher uncontrolled rate of respiration to be observed after addition of proton translocator, indicating that Ca^{2+} -induced damage

Fig. 1. The effect of acetoacetate on respiration and Ca²⁺ accumulation in the presence and absence of ATP plus oligomycin

Mitochondria (1.5 mg of protein/ml of incubation) were incubated at pH 7.0 and 30°C in a medium containing 75 mM-NaCl, 10 mM-Tes (sodium salt), 2 mM-succinate (sodium salt), 32 μ M-albumin (bovine, fraction V), 1 μ M-rotenone, 2 mM-nitrilotriacetate (sodium salt), 2 mM-P_i (sodium salt), 105 μ M-Ca²⁺ (including endogenous Ca²⁺), with the further additions of: a, control; b, 0.2 mM-ATP (sodium salt) and 2 μ g of oligomycin/ml; c, 2 mM-acetoacetate (lithium salt); d, 2 mM-acetoacetate, 0.2 mM-ATP and 2 μ g of oligomycin/ml. Where indicated 0.4 μ M-carbonylcyanide *p*-trifluoromethoxyphenylhydrazine (FCCP) was added. Respiration and pCa₀²⁺ were determined in parallel.

observed in Fig. 1(a) caused not only uncoupling but also a partial inhibition of respiration. Clearly ATP and oligomycin can exert a significant protective role under these conditions (Rossi & Lehninger, 1964). ATP, ADP or oligomycin, alone or in combination, were also able to exert a protective effect (results not shown).

The inclusion of 2 mM-acetoacetate in the initial incubation medium (Fig. 1c) increases the respiratory rate, prevents respiratory control from being observed at 5 min and results in the rapid and total release of the Ca²⁺ that had initially been accumulated. Lehninger *et al.* (1978) also observed a linear respiration under similar conditions, although they did not establish whether respiratory control was demonstrable after addition of proton translocator. No spontaneous release of Ca²⁺ was observed when either 2 mM-acetoacetate plus 2 mM-citrate or 2 mM-acetoacetate plus 10 mM- β -hydroxybutyrate were initially present, in agreement with the proposal of Lehninger *et al.* (1978) that the Ca²⁺ release correlates with the NAD⁺/NADH ratio imposed by substrate couples.

In Fig. 1(d), the initial incubation contained not only acetoacetate, but also ATP and oligomycin. It is apparent that these further additions are able to abolish completely the effects of acetoacetate, both on respiration and on Ca²⁺ transport.

Fig. 2. The effect of phosphoenolpyruvate on Ca²⁺ accumulation in the presence and absence of ATP plus oligomycin

Mitochondria were incubated under the basic conditions of Fig. 1, except that the total Ca²⁺ concentration was 150 μ M. In Fig. 2(b) 2 mM-phosphoenolpyruvate (sodium salt) was initially present, and in Fig. 2(c) 2 mM-phosphoenolpyruvate, 0.2 mM-ATP and 2 μ g of oligomycin/ml were initially present.

The effect of acetoacetate on Ca²⁺ retention shows a similarity to that produced by phosphoenolpyruvate (Fig. 2), in that both agents induce a time-dependent efflux that is prevented by ATP and oligomycin. Phosphoenolpyruvate-induced efflux has been shown by Roos *et al.* (1978) to be accompanied by a collapse of $\Delta\psi$, and these authors consequently suggest that this efflux occurs by reversal of the uniporter.

In Fig. 3, the effect of adding acetoacetate to mitochondria that have accumulated Ca²⁺ is shown to be dependent on the extent of this accumulation. When only about 20 nmol of Ca²⁺/mg of protein is accumulated (calculated from the difference between the total Ca²⁺ in the incubation and the extramitochondrial Ca²⁺ required to produce the observed pCa₀²⁺), acetoacetate addition causes no efflux of Ca²⁺, even though the matrix nicotinamide nucleotides become more oxidized (Fig. 3a). Only when the matrix Ca²⁺ is increased to 87 nmol/mg of protein (Fig. 3b) does acetoacetate addition cause Ca²⁺ release. This release is prevented by the initial presence of ATP and oligomycin (Fig. 3c), although these agents do not prevent the nucleotide oxidation.

Acetoacetate-induced Ca²⁺ release was also shown to be a function of P_i concentration. With decreased P_i concentration a higher Ca²⁺ concentration was required before efflux could be observed (results not shown).

In Fig. 4 the time-course of Ca²⁺ accumulation in the presence of acetoacetate is followed isotopically and is compared with the magnitude of $\Delta\psi$ determined in the same incubation. It is apparent that the release of Ca²⁺ from the matrix in the absence of ATP

Fig. 3. The effect of total Ca^{2+} concentration on the release of matrix Ca^{2+} induced by acetoacetate (AcAc) in the presence and absence of ATP and oligomycin

Mitochondria were incubated under the basic conditions of Fig. 1, except that the total Ca^{2+} concentration was $20\text{ }\mu\text{M}$ in Fig. 3(a) and $150\text{ }\mu\text{M}$ in Figs. 3(b) and 3(c). ATP (0.2 mM) and $2\text{ }\mu\text{g}$ of oligomycin/ml were initially present in Fig. 3(c). Where indicated 2 mM -acetoacetate (lithium salt) was added. pCa_0^{2+} and nicotinamide nucleotide fluorescence were determined in parallel. The Ca^{2+} traces were corrected for the slight electrode artefact caused by the addition of Li^+ .

Fig. 4. The effect of ATP plus oligomycin on the accumulation of Ca^{2+} and the maintenance of membrane potential in the presence of acetoacetate

Mitochondria were incubated in the presence of $[^3\text{H}]$ methyltriphenylphosphonium bromide and $^{45}\text{Ca}^{2+}$ as described in the Experimental section. Symbols O, incubation in the presence of 2 mM -acetoacetate; ●, incubation in the presence of 2 mM -acetoacetate, 0.2 mM -ATP and $2\text{ }\mu\text{g}$ of oligomycin/ml.

and oligomycin correlates closely with the collapse of the membrane potential, and that ATP and oligomycin, by preventing the time-dependent collapse of $\Delta\psi$, also prevent the acetoacetate-induced efflux of Ca^{2+} . When 2 mM - β -hydroxybutyrate was substituted for acetoacetate, $\Delta\psi$ was maintained

above 130 mV , and Ca^{2+} was retained for the duration of the experiment in both the presence and absence of ATP and oligomycin (results not shown).

In Fig. 3 it is shown that the release of Ca^{2+} catalysed by acetoacetate required a threshold level of Ca^{2+} in the matrix. The experiment depicted in Fig. 5 demonstrates that the collapse of the mitochondrial membrane potential shows a similar threshold. Under control conditions, when the matrix nicotinamide nucleotides are maintained reduced by the presence of 2 mM - β -hydroxybutyrate, $\Delta\psi$ was maintained for at least 4 min unless the total Ca^{2+} in the incubation exceeded 170 nmol/mg of protein. The presence of ATP and oligomycin prevents even this decrease in potential, regardless of the oxidation state of the nicotinamide nucleotides. In contrast, when acetoacetate alone is present, potential collapse occurs at values as low as 40 nmol of Ca^{2+}/mg of protein. It is clear therefore that the effect of acetoacetate is to greatly sensitize the mitochondria to the collapse of membrane potential induced by Ca^{2+} , with the result that Ca^{2+} efflux probably occurs by reversal of the uniporter. It should be noted that although the acetoacetate-induced efflux is not inhibited by Ruthenium Red (Lehninger *et al.*, 1978), this does not preclude efflux via the uniporter, as Ruthenium Red has been shown to be an ineffective inhibitor of net Ca^{2+} efflux under conditions of low membrane potential (Puskin *et al.*, 1976; Pozzan *et al.*, 1977). The demonstration by Fiskum & Lehninger (1979) of net $2\text{H}^+/\text{Ca}^{2+}$ ex-

Fig. 5. The effect of total Ca²⁺ concentration, nicotinamide nucleotide reduction and ATP plus oligomycin on the maintenance of membrane potential

Mitochondria were incubated for the determination of membrane potential as described in the Experimental section. Initial additions were: ○, 2mM-β-hydroxybutyrate; ●, 2mM-β-hydroxybutyrate, 0.2mM-ATP and 2μg of oligomycin/ml; △, 2mM-acetoacetate; ▲, 2mM-acetoacetate, 0.2mM-ATP and 2μg of oligomycin/ml. Membrane potential was determined 4 min after addition of the mitochondria.

change under these conditions is consistent with reversal of the uniporter charge compensated by a proton leak.

Conclusion

It is clear from the results presented above that the redox state of the matrix nicotinamide nucleotides greatly influences the ability of mitochondria to retain their integrity when Ca²⁺ is accumulated within the matrix, and that this parameter must be added to the growing number of factors, listed in the introduction, that sensitize the mitochondria to the deleterious effects of Ca²⁺ accumulation. The reasons for this sensitization are unknown. It has been suggested that intramitochondrial adenine nucleotides protect against Ca²⁺-induced damage by inhibiting crystallization of accumulated Ca²⁺ and P_i (see Lehninger, 1970), and it is conceivable that a similar function is served by reduction of intramitochondrial nicotinamide nucleotides. The effect of nicotinamide nucleotide oxidation could be indirect, by decreasing the concentrations of substrates of NAD(P)⁺-linked dehydrogenases, such as the tricarboxylic acids, which might perform a role in inhibiting crystallization.

Although it is clearly important to rationalize these effects, to understand the conditions which must prevail in the matrix for Ca²⁺ to be accumulated without disrupting the other bioenergetic functions of the mitochondria, it is most unlikely that any of these effectors plays a significant regulatory

role in the maintenance of cytosolic Ca²⁺ concentrations *in vivo*. Not only is the Ca²⁺ efflux induced by these agents complete and uncontrolled, but also it would be predicted that the presence of millimolar concentrations of adenine nucleotides in the cytosol (Akerboom *et al.*, 1978) would effectively prevent such damage from occurring.

To be able to distinguish the true independent efflux pathway from these effects, it would seem necessary to demonstrate first that efflux occurs when the membrane potential is maintained at a high value, and secondly that efflux persists in the presence of sufficient external adenine nucleotide to inhibit mitochondrial disruption. To date the only mechanism that satisfy both of these criteria are the Na⁺-dependent efflux pathway of heart and brain mitochondria, and the corresponding Na⁺-independent pathway of liver mitochondria.

References

- Akerboom, T. P. M., Bookelmann, H., Zuurendonk, P. F., van der Meer, R. & Tager, J. M. (1978) *Eur. J. Biochem.* **84**, 413–420
- Akerman, K. E. O. (1978) *Arch. Biochem. Biophys.* **189**, 256–262
- Asimakis, G. K. & Sordahl, L. A. (1977) *Arch. Biochem. Biophys.* **179**, 200–210
- Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M. & Dell'Antone, P. (1977) *FEBS Lett.* **78**, 21–24
- Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kuliene, V. V., Levitsky, D. O., Liberman, E. A., Severina, I. I. & Skulachev, V. P. (1970) *Biochim. Biophys. Acta* **216**, 13–21
- Bygrave, F. L. (1978) *Biol. Rev. Cambridge Philos. Soc.* **53**, 43–79
- Chappell, J. B. & Crofts, A. R. (1965) *Biochem. J.* **95**, 378–392
- Chudapongse, P. & Haugaard, N. (1973) *Biochim. Biophys. Acta* **307**, 599–606
- Crompton, M. & Heid, I. (1978) *Eur. J. Biochem.* **91**, 599–608
- Crompton, M., Capano, M. & Carafoli, E. (1976) *Eur. J. Biochem.* **69**, 453–462
- Crompton, M., Moser, R., Lüdi, H. & Carafoli, E. (1978) *Eur. J. Biochem.* **82**, 25–31
- Ernster, L. (1956) *Exp. Cell Res.* **10**, 704–720
- Fiskum, G. & Lehninger, A. L. (1979) *J. Biol. Chem.* **254**, 6236–6239
- Gazzotti, P. (1975) *Biochem. Biophys. Res. Commun.* **67**, 634–638
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–760
- Heaton, G. M. & Nicholls, D. G. (1976) *Biochem. J.* **156**, 635–646
- Hutson, S. M. (1977) *J. Biol. Chem.* **252**, 4539–4545
- Lehninger, A. L. (1970) *Biochem. J.* **119**, 129–138
- Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 259–320
- Lehninger, A. L., Vercesi, A. & Bababunmi, E. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1690–1694
- McIntyre, H. J. & Bygrave, F. L. (1974) *Arch. Biochem. Biophys.* **165**, 744–748

- Meisner, H. & Klingenberg, M. (1968) *J. Biol. Chem.* **243**, 3631–3639
- Nicholls, D. G. (1978a) *Biochem. J.* **170**, 511–522
- Nicholls, D. G. (1978b) *Biochem. J.* **176**, 463–474
- Nicholls, D. G. & Scott, I. D. (1979) in *Functional and Molecular Aspects of Biomembrane Transport* (Klingenberg, M., Palmieri, F. & Quagliariello, E., eds.), pp. 179–182, Elsevier, Amsterdam
- Nicholls, D. G. & Scott, I. D. (1980) *Biochem. J.* **186**, 833–839
- Out, T. A., Kemp, A. & Souverijn, J. H. M. (1971) *Biochim. Biophys. Acta* **245**, 299–304
- Peng, C. F., Price, D. W., Bhuvaneswaran, C. & Wadkins, C. L. (1974) *Biochem. Biophys. Res. Commun.* **56**, 134–141
- Pozzan, T., Bragadin, M. & Azzone, G. F. (1977) *Biochemistry* **16**, 5618–5625
- Prpić, V., Spencer, T. L. & Bygrave, F. L. (1978) *Biochem. J.* **176**, 705–714
- Puskin, J. S., Gunter, T. E., Gunter, K. K. & Russell, P. R. (1976) *Biochemistry* **15**, 3834–3842
- Roos, I., Crompton, M. & Carafoli, E. (1978) *FEBS Lett.* **94**, 418–421
- Rossi, C. S. & Lehninger, A. L. (1964) *J. Biol. Chem.* **239**, 3971–3980
- Scott, I. D. & Nicholls, D. G. (1980) *Biochem. J.* **186**, 21–33
- Sul, H. S., Shrago, E. & Shug, A. L. (1976) *Arch. Biochem. Biophys.* **172**, 230–237