RUTHENIUM RED SENSITIVE AND INSENSITIVE CALCIUM TRANSPORT IN RAT LIVER AND EHRLICH ASCITES TUMOR CELL MITOCHONDRIA

G. FISKUM and R. S. COCKRELL

Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, MO 63104, USA

Received 13 June 1978

1. Introduction

Calcium affects numerous aspects of cellular metabolism [1,2] and the transport of Ca^{2+} by mitochondria is an important factor in regulating the intracellular distribution of this ion [1,3]. It has been suggested that certain metabolic abnormalities of neoplasms are the consequence of massive Ca^{2+} uptake by mitochondria [2]. The factors underlying the capacity of tumor mitochondria to accumulate large amounts of Ca^{2+} are presently unclear. This is partially due to our lack of understanding of certain features of Ca^{2+} transport by mitochondria from normal tissues.

It is generally accepted that Ca^{2+} uptake is electrogenic and proceeds via a permease which can be specifically inhibited by substances such as ruthenium red [4]. There is however lack of agreement regarding the mechanism of passive Ca^{2+} efflux from mitochondria, e.g., whether Ca^{2+} release occurs via simple reversal of the Ca^{2+} permease or involves other pathways [5–11]. The objectives of the present studies were: (a) to determine if Ca^{2+} efflux is altered in tumor mitochondria and thus a factor in their unusual facility for accumulating this ion and, (b) to further elucidate possible efflux mechanisms for normal and tumor mitochondria.

Rat liver mitochondria and those of Ehrlich ascites tumor cells were compared. It was found that Ca^{2+} efflux proceeded via ruthenium red sensitive and insensitive pathways. The latter mode of transport was much less active in tumor mitochondria. Evidence for one possible mechanism of ruthenium red insensitive Ca^{2+} efflux, Ca^{2+} for H^+ exchange [12,13], was provided by the demonstration that metabolically inhibited liver mitochondria can accumulate Ca^{2+} by means of an artificially induced pH gradient. Furthermore, this activity was not detectable in tumor mitochondria.

2. Experimental

2.1. *Preparation of mitochondria*

A slight modification of the procedure of Schneider [14] was employed for isolating rat liver mitochondria. Mitochondria from Ehrlich ascites tumor cells were prepared by the method described by Reynafarje et al. [15] for isolating mitochondria from L1210 ascitic leukemia cells. Protein was assayed by the Lowry method [16].

2.2. *Measurements of calcium flux*

Ca^{2+} fluxes were determined spectrophotometrically by the murexide indicator technique [17]. The suspending media contained 250 mM sucrose, 10 mM Tris–MOPS (pH 7.2), 5 mM Tris-succinate, 2.5 mM Tris-phosphate, 30 μM murexide and oligomycin (6 μg/ml). The temperature was maintained at 25°C and the mitochondria were pre-treated with rotenone (0.2 μg/mg).

3. Results

3.1. *Calcium efflux*

Mitochondria were preloaded with Ca^{2+} aerobically and passive efflux initiated with uncoupling agent.

The rate of Ca^{2+} release from tumor mitochondria was only one-third the rate for rat liver (table 1). Whereas ruthenium red sensitive efflux was nearly identical for the two types of mitochondria, the ruthenium red insensitive Ca^{2+} efflux of liver mitochondria was 18 times more rapid than that of tumor mitochondria. The differences in rates of ruthenium red insensitive Ca^{2+} release might reflect either a difference in permeability or internal free Ca^{2+} concentration.

The ionophore A23187 which catalyzed neutral Ca^{2+} for H^+ exchange [18] was used to test for differences in the intramitochondrial Ca^{2+} concentration. As shown in table 1 the release rate in the presence of A23187 was actually greater for tumor than liver mitochondria. This indicated that tumor mitochondria possessed a lower ruthenium red insensitive Ca^{2+} permeability than rat liver rather than simply differing in internal free Ca^{2+}.

The intramitochondrial Ca^{2+} level was however a factor in ruthenium red insensitive Ca^{2+} translocation. If Ca^{2+} loading of tumor mitochondria exceeded 600 neq/mg, ruthenium red insensitive efflux rose sharply (fig.1). At 1200 neq/mg, Ca^{2+} release by this pathway was as rapid as for rat liver preloaded with 300 neq Ca^{2+}/mg. This apparent regulation of ruthenium red insensitive Ca^{2+} permeability could reflect damage to the inner mitochondrial membrane by high levels of internal Ca^{2+}. Uncoupling (e.g. increased H^+ conductance) would be expected if this were the case. However basal respiration of liver mitochondria was increased no more than 15% at Ca^{2+}

Table 1
Comparison of Ca^{2+} efflux values for rat liver and tumor mitochondria

Parameter	Rat Liver	Tumor
Efflux	165	56
R.R. sensitive efflux	57	50
R R. insensitive efflux	108	6
Efflux + A23187	570	750

After completion of Ca^{2+} uptake (300 neq/mg), Ca^{2+} efflux was initiated with FCCP (4 μM). All flux values are expressed in neq Ca^{2+}/min·mg. When included, ruthenium red (4 μM) was added immediately before FCCP, and A23187 (2 μg/ml) simultaneously with FCCP. The final mitochondrial protein concentration was 4 mg/ml (R.R. = Ruthenium Red)

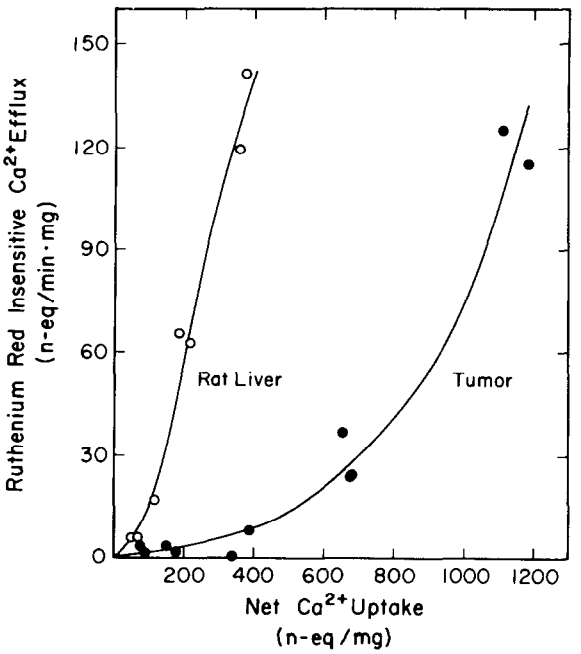

Fig.1. Ruthenium red insensitive Ca^{2+} efflux as a function of the Ca^{2+} load. The conditions were as described in the legend to table 1.

levels associated with rapid ruthenium red insensitive Ca^{2+} efflux (cf. table 1) and that of tumor mitochondria was actually somewhat greater under these conditions yet efflux was much slower. Therefore no simple relationship between the degree of uncoupling and the rate of ruthenium red insensitive Ca^{2+} efflux was evident (see ref. 19 and section 4).

3.2. *Ruthenium red insensitive Ca^{2+} transport driven by a pH gradient*

It was previously postulated that ruthenium red insensitive mitochondrial Ca^{2+} transport might proceed by a different mechanism than that of ruthenium red sensitive transport, e.g. it could involve exchange with protons [12,13]. If such a mechanism existed a pH gradient established with dianemycin (an ionophore which can catalyze release of endogenous K^+ ions and proton uptake) should drive Ca^{2+} uptake by metabolically inhibited mitochondria. This was demonstrated as shown in fig.2.

Calcium was added to mitochondria in the presence of ruthenium red plus uncoupler which prevented

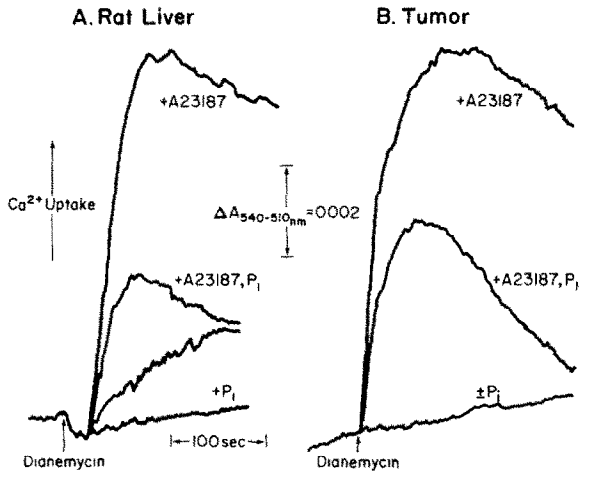

Fig.2. Calcium uptake driven by a pH gradient. The medium was as described in section 2.2 except succinate was omitted and phosphate included as indicated. Prior to addition of dianemycin (0.4 μg/ml), FCCP (2 μM), ruthenium red (4 μM) and $CaCl_2$ were added sequentially. In A, the protein concentration was 4.6 mg/ml, Ca^{2+} added 20 neq/mg and, when included, A23187, 0.4 μg/ml. In B, 3.7 mg protein/ml, 100 neq Ca^{2+}/mg and A23187, 2 μg/ml, was employed.

energy-linked uptake. Upon addition of dianemycin, Ca^{2+} was accumulated by rat liver (fig.2A) but not tumor mitochondria (fig.2B) although they released comparable amounts of K^+ (measured with a K^+ sensitive electrode). The ionophore A23187 enhanced pH gradient driven Ca^{2+} flux in rat liver mitochondria and was required for Ca^{2+} uptake by tumor mitochondria. Thus Ca^{2+} accumulation could occur in tumor mitochondria given the appropriate permeability. Phosphate inhibited these Ca^{2+} movements which provided further evidence that they were driven by the pH gradient set up by dianemycin. This inhibition most likely resulted from competition by the phosphate/OH^- transport system [20] for the imposed pH gradient.

4. Discussion

It has been reported that there is no substantial difference between the kinetics for Ca^{2+} uptake by tumor and rat liver mitochondria [2]. Yet under most conditions the maximum net uptake of Ca^{2+} by tumor mitochondria is much greater [15,21,22]. In the present studies a significant difference in the rate of release of accumulated Ca^{2+} from normal and tumor mitochondria was demonstrated. Since Ca^{2+} uptake rates for normal and tumor mitochondria are comparable, the comparatively slow Ca^{2+} efflux from tumor mitochondria appears to be a significant factor in their greater uptake capacity although it need not be solely responsible.

The most significant difference in Ca^{2+} efflux for the two types of mitochondria was the relative inactivity of the ruthenium red insensitive pathway in tumor mitochondria. In contrast, ruthenium red sensitive Ca^{2+} transport either in the case of influx [2] or passive efflux (table 1) appears to be unaltered in tumor mitochondria.

The mechanism of ruthenium red insensitive Ca^{2+} efflux is not known. Evidence presented here indicates a pH gradient can drive ruthenium red insensitive Ca^{2+} translocation. Uncoupling agent stimulates this transport of Ca^{2+} (unpublished experiments) which suggests it is electrogenic, for example, a Ca^{2+}/H^+ antiport [8]. Uncoupling agent is also necessary for rapid ruthenium red insensitive Ca^{2+} efflux (see also refs. 8,9) which suggests it too is electrogenic. This is reminiscent of electrogenic Ca^{2+}/Na^+ exchange of heart mitochondria [10,11] although this activity appears to be negligible in rat liver mitochondria [10]. Other explanations of ruthenium red insensitive Ca^{2+} efflux are not excluded by results presented here, for example, electrophoretic Ca^{2+} efflux indirectly coupled to the counterflow of protons through a H^+ 'leak' [19]. However the recent report that oxidation of pyridine nucleotides accelerates ruthenium red insensitive release of mitochondrial Ca^{2+} [23] with no apparent effect upon membrane integrity or coupling does not comply with a simple proton 'leak' mechanism [19]. These studies are interpreted in terms of separate pathways for Ca^{2+} influx and efflux [23] such as inferred from the results presented here. Further studies of regulatory factors such as pyridine nucleotides [23] in liver and tumor mitochondria are necessary to clarify the mechanism of Ca^{2+} efflux for which the pH driven Ca^{2+} uptake system may provide one useful approach.

Different modes of mitochondrial Ca^{2+} influx and efflux may be important in cellular Ca^{2+} homesostasis particularly since they may be regulated separately

[23]. The fact that these differ quantitatively in rat liver and tumor mitochondria could bear upon possible alterations in steady state cytoplasmic Ca^{2+} levels in hepatocytes and tumor cells [2]. Studies are under way to determine whether mitochondrial Ca^{2+} transport has the same characteristics in situ as in vitro and what bearing this has on the metabolism of normal and neoplastic cells.

Acknowledgements

This investigation was supported by funds from Public Health Service Grant No. CA11766 from the National Cancer Institute; G. F. is the recipient of a N.I.H. predoctoral fellowship (GM 00446).

References

[1] Rasmussen, H. (1970) Science 170, 404–412.
[2] Bygrave, F. L. (1976) in: Control Mechanisms in Cancer (Criss, W. E., Ono, T. and Sabine, J. R., eds) pp. 411–423, Raven Press, New York.
[3] Lehninger, A. L. (1970) Biochem. J. 119, 129–138.
[4] Moore, C. L. (1970) Biochem. Biophys. Res. Commun. 42, 298–305.
[5] Vasington, F. D., Gazotti, P., Tiozzo, R. and Carafoli, E. (1972) Biochem. Biophys. Acta 256, 43–54.
[6] Rossi, C. S., Alexandre, A. and Rossi, C. R. (1974) FEBS Lett. 43, 349–352.
[7] Sordahl, L. A. (1974) Arch. Biochem. Biophys. 167, 104–115.
[8] Puskin, J. S., Gunter, T. E., Gunter, K. K. and Russell, P. R. (1976) Biochemistry 15, 3834–3842.
[9] Pozzan, T. and Azzone, G. F. (1976) FEBS Lett. 71, 62–66.
[10] Crompton, M., Capano, M. and Carafoli, E. (1976) Eur. J. Biochem. 69, 453–462.
[11] Crompton, M., Kunzi, M. and Carafoli, E. (1977) Eur. J. Biochem. 79, 549–558.
[12] Cockrell, R. S. (1976) Fed. Proc. 35, 223.
[13] Fiskum, G. (1976) Fed. Proc. 35, 1509.
[14] Schneider, W. (1948) J. Biol. Chem. 176, 259–266.
[15] Reynafarje, B. and Lehninger, A. L. (1973) Proc. Natl. Acad. Sci. USA 70, 1744–1748.
[16] Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Scarpa, A. (1972) in: Methods in Enzymology, (San Pietro, D. M., ed) vol. XXIV, pp. 343–351, Academic Press, New York.
[18] Reed, P. W. and Lardy, H. A. (1972) J. Biol. Chem. 247, 6970–6977.
[19] Pozzan, T., Bradadin, M. and Azzone, G. F. (1977) Biochemistry 16, 5618–5624.
[20] Chappell, J. B. and Haarhoff, K. N. (1967) in: Biochemistry of Mitochondria, (Slater, E. C., Kaniuga, Z. and Wojtzak, L. eds) pp. 75–91, Academic Press, London.
[21] Thorne, R. F. W. and Bygrave, F. L. (1974) Nature 248, 348–351.
[22] McIntyre, H. J. and Bygrave, F. L. (1974) Arch. Biochem. Biophys. 105, 744–748.
[23] Lehninger, A. L., Vercesi, A. and Bababunmi, E. A. (1978) Proc. Natl. Acad. Sci. USA 75, 1690–1694.