Biochimica et Biophysica Acta, 766 (1984) 549–553
Elsevier

BBA 41582

CALCIUM TRANSPORT BY RAT BRAIN MITOCHONDRIA AND OXIDATION OF 2-OXOGLUTARATE

PHYLLIS A. BERNARD * and RONALD S. COCKRELL **

Edward A. Doisy Department of Biochemistry St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104 (U.S.A.)

(Received March 1st, 1984)
(Revised manuscript received May 21st, 1984)

Key words: Ca^{2+} transport; 2-Oxoglutarate dehydrogenase; Respiration; Brain mitochondria; (Rat)

Contrary to previous reports brain mitochondria have a substantial capacity for net Ca^{2+} uptake (approx. 1.2 μeq. Ca^{2+} per mg protein) providing succinate is the oxidizable substrate. ATP stimulates calcium uptake (to 1.8 μeq. per mg protein), but is not required. The accumulation of Ca^{2+} with NAD-linked substrates is, however, significantly less. With 2-oxoglutarate, very limited Ca^{2+} uptake occurs before respiration is inhibited. At low concentrations (10 μM), Ca^{2+} stimulates the 2-oxoglutarate dehydrogenase activity of detergent solubilized mitochondria. Millimolar [Ca^{2+}] is required for inhibition. Therefore, Ca^{2+} inhibition of 2-oxoglutarate oxidation can explain the low maximum uptake with this substrate, but probably not by directly effecting the dehydrogenase. Hence, the oxidation of 2-oxoglutarate can be either enhanced or suppressed depending upon the net Ca^{2+} accumulated by brain mitochondria.

Introduction

Early studies of Ca^{2+} uptake by brain mitochondria indicated uptake was low with oxidizable substrate compared to ATP [1,2]. Since ATP alone supported appreciable Ca^{2+} uptake, it actually increased uptake only marginally with oxidizable substrate [2]. Later studies with more highly purified brain mitochondria revealed they were capable of significantly greater oxidizable substrate supported Ca^{2+} uptake [3]. The ATP-dependent Ca^{2+} accumulation was about the same as reported previously. Nevertheless, these maximum Ca^{2+} uptake values, 0.3–0.7 μeq. per mg protein [3] were still less than for mitochondria from other tissues [4].

Evaluation of Ca^{2+} transport by a purified preparation of intact rat brain mitochondria [5] was therefore undertaken to determine whether it was more typical. Mitochondria isolated from brain slices whose intracellular [Ca^{2+}] is elevated retain large amounts of endogenous Ca^{2+} [6,7]. It seemed reasonable their maximum capacity should therefore be quite large. The enhanced uptake of Ca^{2+} by tumor cell mitochondria [8–10] has been evoked to explain rapid aerobic glycolysis by neoplastic cells [11,12]. Since brain exhibits rapid aerobic glycolysis [13,14], it was of interest to determine whether the Ca^{2+} uptake capacity of our brain mitochondrial preparation was greater than normal.

It has been demonstrated that the capacity of rat brain mitochondria to accumulate Ca^{2+} is as great as for mitochondria from rat liver. In both cases, however, this is about one-half that of tumor cell mitochondria [10]. Ca^{2+} uptake is substrate dependent, e.g., much less with NAD-linked sub-

* Present address: Department of Neurology, Cornell University School of Medicine, New York, NY, U.S.A.
** To whom correspondence should be addressed.
Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

strates than succinate. This is especially striking with 2-oxoglutarate, where Ca^{2+} accumulation is only one-tenth as great as with succinate. Calcium inhibits respiration with 2-oxoglutarate and high levels of Ca^{2+} inhibit the 2-oxoglutarate dehydrogenase of solubilized mitochondria. The available substrate can therefore stringently limit Ca^{2+} uptake by brain mitochondria. Correspondingly, the extent of Ca^{2+} transport by brain mitochondria can regulate the oxidative metabolism of key metabolites such as 2-oxoglutarate.

Materials and Methods

Brain mitochondria were isolated from the cortices and cerebella of male rats (100–200 g) as described previously [5].

Calcium transport was measured spectrophotometrically with murexide at 540–510 nm by means of an Aminco DW-2 spectrophotometer [15]. Maximum Ca^{2+} uptake was determined from sequential 250 μM additions of Ca^{2+} to mitochondria at concentrations of approx. 1 mg protein per ml.

Oxygen uptake rates were measured polarographically with a Clarke (membrane) electrode [16].

Mitochondrial 2-oxoglutarate: lipoate oxidoreductase (EC 1.2.4.2, oxoglutarate dehydrogenase) activity was determined by dual wavelength spectroscopic measurements of NAD^+ reduction (340–374 nm) at protein concentrations of approx. 50 μg per ml. Mitochondria were treated with rotenone (0.2 μg per mg protein), solubilized with Triton X-100 (1%) and assayed immediately for maximal activity. The reaction media consisted of 50 or 45 mM potassium acetate plus 5 mM KP_i, 3 mM cysteine, 1 mM $MgCl_2$, 2 mM NAD^+ and 0.2 mM thiamine pyrophosphate at pH 7.2 and 25 °C. The reaction was initiated by simultaneous addition of 1 mM Tris-2-oxoglutarate and 60 μM coenzyme A.

Endogenous mitochondrial divalent cation levels were determined with a Perkin Elmer (Model 303) atomic absorption spectrometer on 10% nitric acid extracts.

Mitochondrial protein was determined by the method of Lowry et al. [17], with bovine serum albumin as the standard.

Materials

Male rats (Wistar strain) were purchased from Hilltop Laboratories, Scottsdale, PA. Mannitol, murexide, thiamine pyrophosphate, oligomycin, atractylate, rotenone, pyruvate, 2-oxoglutarate, fatty acid-free bovine serum albumin, ATP, NAD, coenzyme A and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were purchased from Fisher Scientific Co., Fair Lawn, NJ. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was kindly provided by Dr. P.G. Heytler, E.I. Dupont de Nemours and Co., Wilmington, DE.

Results

Maximum Ca^{2+} accumulation was first examined with succinate, the substrate most rapidly oxidized by this preparation of brain mitochondria [5]. Albumin more than doubled the extent of Ca^{2+} uptake (Table I) and exogenous Mg^{2+} promoted further Ca^{2+} accumulation (1.21 μeq. Ca^{2+} per mg). Uptake was maximum if ATP was also added (1.81 μeq. per mg protein). Stimulation by ATP was only partially sensitive to oligomycin or attractylate (either individually or combined). Maximum Ca^{2+} accumulation under these conditions was at least two times greater than reported

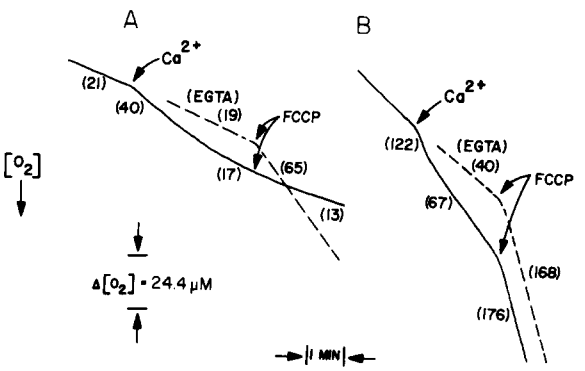

Fig. 1. Calcium effects on the respiration of brain mitochondria. Mitochondria (1.1 mg/ml) were suspended in medium containing 300 mM mannitol, 15 mM potassium glycylglycine and 2.5 mM Tris-P_i at pH 7.2. The substrate was either 5 mM Tris-2-oxoglutarate (A) or 5 mM Tris-succinate (plus 0.2 μg rotenone per mg protein) in (B). Other additions are indicated on the figure: Tris-EGTA (0.2 mM), $CaCl_2$ (97 μM), FCCP (0.1 μM). The values in parentheses are the respiration rates (ng atoms O/min per mg protein).

previously (with oxidizable substrate on ATP alone, Table I). These values are quite similar to those obtained for rat liver mitochondria with succinate (plus rotenone). Our preparations of liver mitochondria accumulate 0.68 ± 0.15 (no additions). 0.82 ± 0.15 (+bovine serum albumin), 1.58 ± 0.20 (+bovine serum albumin, Mg^{2+}) and 2.23 ± 0.17 μeq. Ca^{2+} per mg protein (+bovine serum albumin, Mg^{2+}, ATP). Although the extent of stimulation by bovine serum albumin is somewhat less for rat liver than for brain mitochondria, other differences are not remarkable. These maximum uptake values for brain and liver mitochondria are approx. one-half the comparable values for Ehrlich ascites tumor cell mitochondria [10]. The uptake of Ca^{2+} by brain mitochondria depended very much upon the oxidizable substrate employed (whether ATP was present or not). The uptake with NAD-linked substrates was appreciably less than with succinate with a rank order of pyruvate > glutamate $\gg$ 2-oxoglutarate (0.91, 0.45 and 0.18 μeq. per mg protein, respectively; $n = 3$). It is noteworthy that omitting rotenone decreased the uptake with succinate to one-third (0.51 vs. 1.50 μeq. per mg protein, Table I). Failure to include rotenone with succinate might explain the lower Ca^{2+} uptake values reported previously [2]. Similarly, the use of NAD-linked substrates [3] could have been one factor in obtaining lower uptake values (plus the effects of bovine serum albumin, see Discussion). Calcium uptake with 2-oxoglutarate was markedly less than with the other substrates tested. An explanation for the limited Ca^{2+} accumulation with 2-oxoglutarate was evident from the influence of Ca^{2+} on respiration.

With 2-oxoglutarate as substrate, Ca^{2+} caused a modest stimulation of respiration. This progressed to an inhibited state unaffected by uncoupling agent, FCCP (Fig. 1A). This behavior contrasts with the more typical effects of Ca^{2+} observed with succinate, i.e., a cycle of stimulation followed by slight uncoupling, and maximal stimulation by uncoupling agent (Fig. 1B). These results indicated Ca^{2+} may inhibit oxoglutarate dehydrogenase. This possibility was tested by assaying for direct effects of Ca^{2+} on the oxoglutarate dehydrogenase activity of detergent solubilized mitochondria. The results for a broad range of Ca^{2+} concentrations are provided in Fig. 2.

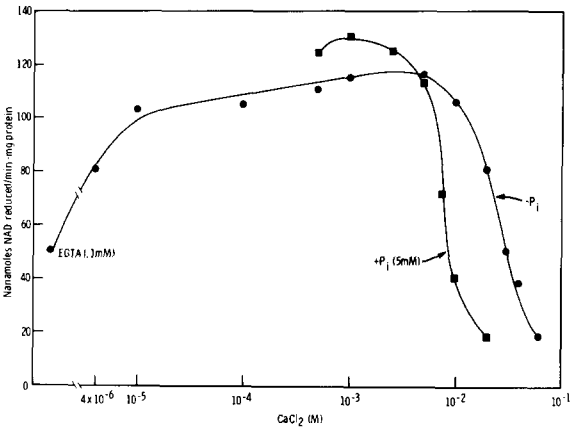

Fig. 2. Effect of Ca^{2+} on the oxoglutarate dehydrogenase activity of brain mitochondria. Oxoglutarate dehydrogenase activities were determined as described in the Materials and Methods section with Tris-EGTA or the indicated concentrations of $CaCl_2$. Values for the series without phosphate (circles) and with phosphate (squares) are averages for five and three mitochondrial preparations, respectively. Standard deviations were 19% or less of the value represented by each point.

TABLE I

MAXIMUM Ca^{2+} UPTAKE BY BRAIN MITOCHONDRIA

Calcium uptake was monitored spectrophotometrically as described in the Materials and Methods section with 80 μM murexide. The suspending medium contained 300 mM mannitol, 15 mM Tris-glycylglycine and 2.5 mM Tris-P_i at pH 7.2 plus either Tris-succinate (5 mM) or Tris-ATP (0.25 mM). Mitochondria were pretreated with rotenone (0.2 μg per mg protein) and added to a final concentration of approx. 1 mg protein per ml. Further additions prior to adding mitochondria are indicated: bovine serum albumin (BSA) (0.08%), $MgCl_2$ (3 mM), Tris-ATP (0.25 mM), oligomycin (10 μg per mg protein) and atractylate (25 μM). $CaCl_2$ was added as 250 μM pulses after completion of the previous uptake cycle until no uptake or spontaneous release occurred, corresponding to maximum uptake for that particular condition. Individual cycles lasted approx. 1 min and therefore from 5 to 10 minutes was required for maximum Ca^{2+} loading. The cuvette was purged with air periodically to insure anaerobiasis did not occur. The values are means ($\pm$S.D.) for three to five mitochondrial preparations.

Addition(s)	Ca^{2+} Uptake (μeq. per mg protein)
Succinate (+rotenone)	0.33 ± 0.12
Succinate, BSA	0.79 ± 0.24
Succinate, BSA, Mg^{2+}	1.21 ± 0.38
Succinate, BSA, Mg^{2+}, ATP	1.81 ± 0.50
Succinate, BSA, Mg^{2+}, ATP, oligomycin, atractylate	1.50 ± 0.35
ATP (+rotenone), BSA, Mg^{2+}	1.50 ± 0.48
ATP (+rotenone), BSA, Mg^{2+}, oligomycin, atractylate	0.07 ± 0.02

TABLE II

INFLUENCE OF Mg^{2+} ON THE OXOGLUTARATE DEHYDROGENASE ACTIVITY OF BRAIN MITOCHONDRIA

The dehydrogenase was assayed essentially as described in the Materials and Methods section, except $MgCl_2$ and $CaCl_2$ were added as indicated. The assay medium contained 45 mM potassium acetate and 5 mM KP_i. The values are means for three separate preparations. Standard deviations were 16% or less of the indicated values.

	$[Mg^{2+}]$ (mM)	$[Ca^{2+}]$ (mM)	Oxoglutarate dehydrogenase activity (% maximal activity)
A	1	0.01	99
	5	0.01	100
	10	0.01	98
	30	0.01	47
	50	0.01	14
B	1	5	96
	20	0.01	85
	20	5	34

Increasing Ca^{2+} from approx. 10^{-8} M (with EGTA) to 10^{-5} M doubled brain oxoglutarate dehydrogenase activity. Only at very high $[Ca^{2+}]$, 10–60 mM, did inhibition occur. Certain ions present endogenously in intact mitochondria were tested to determine whether or not they influenced the concentration dependence of Ca^{2+} inhibition. Phosphate, the major endogenous anion and coion accumulated with Ca^{2+}, shifted inhibition to somewhat lower Ca^{2+} levels, i.e., 50% inhibition at 8 mM Ca^{2+} (Fig. 2). Magnesium was also tested, since it is required for oxoglutarate dehydrogenase activity [18].

Although Mg^{2+} is required for enzyme activity, higher concentrations were found to inhibit much the same as Ca^{2+}. These results are summarized in Table II (series A). Interestingly, it was found that instead of antagonizing Ca^{2+} inhibition, the combination of Mg^{2+} and Ca^{2+} inhibited synergistically (Table II, Series B). At either 5 mM Ca^{2+} or 20 mM Mg^{2+}, the dehydrogenase was effected very little whereas in combination, Ca^{2+} plus Mg^{2+} inhibited about 70%. It should be noted that no comparable inhibition was observed with monovalent cations over the same ionic strength range. Nevertheless, despite the potentiation of Ca^{2+} inhibition by P_i (Fig. 2) and synergistic action of Mg^{2+}, several millimolar Ca^{2+} was required for significant dehydrogenase inhibition (e.g., 5 mM Ca^{2+} – for 70% inhibition, Table IIB). These results indicated Ca^{2+} inhibition of respiration with oxoglutarate (Fig. 1) was unlikely to be due to a direct effect on the dehydrogenase.

Discussion

In the present studies it has been shown that brain mitochondria are able to accumulate Ca^{2+} to the same extent as mitochondria from rat liver. The extent of Ca^{2+} accumulation was much less if succinate was employed without rotenone (Table I). The previously reported requirement for ATP [2] might have been to promote succinate oxidation in the absence of rotenone which may or may not be attributable to prevention of oxalacetate inhibition [19,20]. ATP alone did support significant Ca^{2+} uptake in previous studies, although it was about one-half the values obtained here (Table I) [2,3]. Albumin was not used in the measurements of Lazarewicz et al. [3] which may explain this difference, since bovine serum albumin exerted a significant (2.5-fold) stimulatory effect on Ca^{2+} uptake (Table I).

The very low Ca^{2+} uptake with 2-oxoglutarate as substrate could be due to inhibition of the dehydrogenase by Ca^{2+}, but a direct effect requires several millimolar Ca^{2+} which is unlikely to occur even if localized concentrations in the region of the dehydrogenase greatly exceeded those in the matrix. It is more likely that Ca^{2+} complexes and/or promotes leakage of pyridine nucleotides [21] and oxoglutarate dehydrogenase is particularly susceptible to cofactor loss. In any event, the present studies provide an added dimension to the role of Ca^{2+} in regulation of 2-oxoglutarate metabolism. At physiological Ca^{2+} levels, the dehydrogenase is stimulated (0.01–10 μM; Fig. 2) as in mitochondria from other tissues [22,23] and the isolated enzyme [24]. State 3 respiration with Ca^{2+} or FCCP was blocked completely (Fig. 1A) at 40–50 neq. Ca^{2+} per mg protein (data not presented). This is only a doubling of the endogenous Ca^{2+} for these preparations of brain mitochondria (37 ± 11 neq. Ca^{2+} per mg; mean value for three preparations). Therefore, within ranges of total matrix Ca^{2+} that may prevail in vivo, Ca^{2+} can

promote or attenuate oxidation of this key intermediate of the tricarboxylic acid cycle.

It should be mentioned that the 2-oxoglutarate dehydrogenase of rat-liver mitochondria and coupled respiration with oxoglutarate are also inhibited by Ca^{2+} [25]. In turn, the substrate dependence of Ca^{2+} uptake by brain and liver mitochondria is nearly the same with the notable exception of pyruvate. Maximum Ca^{2+} uptake by brain mitochondria with pyruvate (plus Mg^{2+} and ATP) is about 1.0 μeq. per mg protein, whereas it is about 0.2 for liver (data not presented). Liver and other types of mitochondria exhibit limited Ca^{2+} uptake with various NAD-linked substrates [26–28] which, as discussed for oxoglutarate oxidation, may be attributable to Ca^{2+}-induced pyridine nucleotide leakage and/or complexing of nucleotides by Ca^{2+} [21]. Alternatively, Ca^{2+} may induce metabolic transformations of pyridine nucleotides, particularly $NAD(P)^+$ [29,30]. Hence, the ability of brain mitochondria to accumulate large amounts of Ca^{2+} and maintain respiration with pyruvate is unusual. Pyruvate is the major oxidizable substrate in brain as the principal product of glucose utilization accompanying rapid aerobic glycolysis. These products account for 80–90% of this tissue's respiration [31]. Although exogenous K^+ [32] and perhaps endogenous K^+ [5] stimulate pyruvate oxidation by brain mitochondria, an insensitivity to Ca^{2+} inhibition may also be important for rapid utilization of this substrate.

Acknowledgements

This investigation was supported by funds from Public Health Service Grant No. CA 11766 from the National Cancer Institute. These studies were performed by P.A.B. during tenure of a NIH predoctoral fellowship (GM 00446) as part of a dissertation submitted for the Ph.D. degree.

References

1 Lust, W.D. and Robinson, J.D. (1969) J. Neurobiol. 1, 303–316
2 Tjioe, S., Bianchi, C. and Haugaard, N. (1970) Biochem. Biophys. Acta 216, 270–273
3 Lazarewicz, J.W., Haljamae, H. and Hamberger, A. (1974) J. Neurochem. 22, 33–45
4 Carafoli, E. and Lehninger, A.L. (1971) Biochem. J. 122, 681–690
5 Bernard, P.A. and Cockrell, R.S. (1979) Biochim. Biophys. Acta 548, 173–186
6 Bourke, R.S. and Tower, D.B. (1966) J. Neurochem. 13, 1099–1117
7 Stahl, W.L. and Swanson, P.D. (1969) J. Neurochem. 16, 1553–1563
8 Thorne, R.F.W. and Bygrave, F.L. (1973) Biochem. Biophys. Res. Commun. 50, 294–299.
9 Reynafarje, B. and Lehninger, A.L. (1973) Proc. Natl. Acad. Sci. USA 70, 1744–1748
10 Cockrell, R.S. (1981) Arch. Biochem. Biophys. 212, 443–451
11 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
12 Bygrave, F.L. (1978) Biol. Rev. 53, 43–79
13 Aisenberg, A.C. (1961) The Glycolysis and Respiration of Tumors, Academic Press, New York
14 Himwich, H.E. (1976) Brain Metabolism and Cerebral Disorders, pp. 11–32, Spectrum Publications Inc., New York
15 Scarpa, A. (1972) Methods Enzymol. 24, 343–351
16 Estabrook, R. (1967) Methods Enzymol. 10, 41–47
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randell, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Sanadi, D.R. (1963) in The Enzymes (Boyer, P.D., Lardy, H., and Myrback, K., eds.), Vol. 7, pp. 307–351, Academic Press, New York
19 Azzone, G.F. and Ernster, L. (1961) J. Biol. Chem. 236, 1518–1525
20 Schollmeyer, P. and Klingenberg, M. (1961) Biochem. Biophys. Res. Commun. 4, 43–47
21 Vinogradov, A., Scarpa, A. and Chance, B. (1972) Arch. Biochem. Biophys. 152, 646–654
22 Denton, R.M., McCormack, J.G. and Edgell, N.J. (1980) Biochem. J. 190, 107–117
23 Hansford, R.C. (1981) Biochem. J. 194, 721–732
24 McCormack, J.G. and Denton, R.M. (1979) Biochem. J. 180, 533–544
25 Bernard,P.A. (1978). Fed. Proceed. 37, 1614
26 Vasington, F.D. and Murphy, J.V. (1962) J. Biol. Chem. 237, 2670–2677
27 Cittadini, A., Scarpa, A., and Chance, B. (1973) Biochim. Biophys. Acta 291, 246–259
28 Landry, Y. and Lehninger, A.L. (1976) Biochem. J. 158, 427–438
29 Lötscher, H.R., Winterhalter, K.H., Carafoli, E.and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340–4344
30 Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255 9325–9330
31 Elliott, K.A.C. and Wolfe, L.S. (1962) in The Chemistry of Brain and Nerve (Elliott, K.A.C., Page, I.H., and Quastel, J.H., eds.) Neurochemistry, Edn. 2, pp. 177–182, Charles C. Thomas, Springfield, IL
32 Clark, J.B. and Nicklas, W.J. (1970) J. Biol. Chem. 245, 4724–4731