RESPIRATORY CONTROL AND MITOCHONDRIAL MONOVALENT CATION PERMEABILITY OF ISOLATED LIVER CELLS*

W. P. Dubinsky and R. S. Cockrell

Department of Biochemistry

St. Louis University School of Medicine

St. Louis, Mo. 63104

Received November 16,1973

SUMMARY

Uncoupling agent releases the respiratory control of rat hepatocytes to approximately the same degree as in isolated mitochondria indicating that mitochondria *in situ* possess a low H^+ conductance as *in vitro*. Mitochondria also have no detectable natural K^+ conductance since the ionophore, valinomycin, is required for K^+ ions to uncouple. Na^+ but not K^+ or choline inhibits the uncoupled respiration of liver cells. This is consistent with operation of neutral mitochondrial Na^+ for H^+ exchange *in vivo*. These results indicate a considerable similarity between certain functional and permeability properties of mitochondria *in vitro* and *in situ*. These similarities form the basis for discussion of the role of mitochondrial ion transport in metabolic regulation.

INTRODUCTION

The distribution and flow of metabolites between mitochondrial and cytoplasmic compartments are very likely important functions in metabolic regulation. Studies with isolated mitochondria have established that gradients of various anionic metabolites are determined by cation fluxes (1,2). The extent to which such conclusions apply to intact cells depends however upon the similarity between ionic permeabilities of mitochondria *in vitro* and *in situ* which is not known.

The utility of isolated rat liver cells in the study of metabolic regulation has been reasonably well documented particularly by recent studies of gluconeogenesis, glycolysis and hormonal effects since these results agreed with perfusion studies of intact liver (3,4). The influence of intracellular

*This investigation was supported by funds from Public Health Service Grant No. Ca-11766 from the National Cancer Institute and reported in part at the 9th International Congress of Biochemistry, Stockholm, Sweden, July, 1973; W.P.D. is the recipient of a N.I.H. Predoctoral Fellowship (GM 00446-13).

monovalent cations upon liver cell respiration has been examined to obtain information regarding the in situ mitochondrial permeability to these ions. Release of respiratory control was employed as an assay of ionic permeability and as an indicator of the structural integrity of mitochondria in situ. Respiratory control has been treated in some detail since no comparable results with this cell system have been reported previously.

METHODS

Cells were isolated from livers of 200-250 g male Wistar rats by the method of Ingebretson and Wagle (5) except modified by inclusion of 0.8% hyaluronidase in the perfusion medium. The pH of the perfusion medium was maintained at 7.4 with a Radiometer TTT 11b/SBR2c titrimeter. Cells were resuspended at a final concentration of 45-65 mg protein per ml and stored at 0-4° C.

Oxygen utilization was measured polarographically with a Clarke electrode. Protein was determined by the biuret method (6) and Na^+ and K^+ assayed with an Atomic Absorption Spectrometer.

RESULTS

Respiratory Control

Respiratory control was studied in detail since it is one of the most sensitive indicators of the intactness of mitochondria (7). Typical oxygen electrode tracings with fresh isolated cells are shown in Fig. 1. Endogenous respiration was stimulated by uncoupling agent (FCCP)[1] indicative of respiratory control (trace a); substrate was presumably limiting since the stimulation was transient. Basal respiratory rates were increased by various exogenous substrates as were R.C.I. values (trace b of Fig. 1, obtained with β-OH butyrate). Respiratory control depended upon the concentration and

[1]Abbreviations used: FCCP, Carbonylcyanide p-trifluoromethoxyphenylhydrazone; R.C.I., Respiratory Control Index; EGTA, Ethylenebis(oxyethylene-nitrilo) tetraacetic Acid

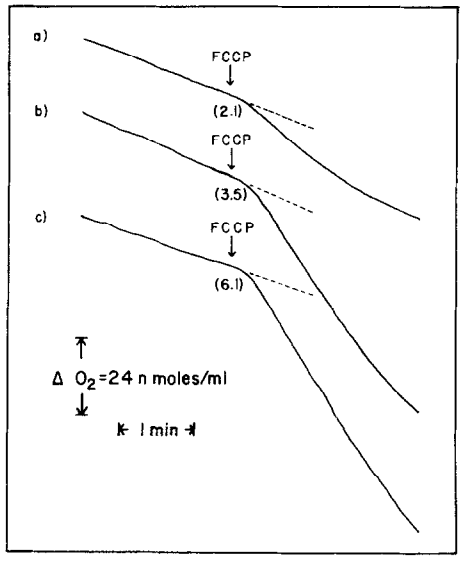

Fig. 1. Fresh isolated cells were suspended in medium consisting of 250 mM sucrose, 25 mM Tris-Cl and 2.5 mM Tris-EGTA at pH 7.2 and 22° C. The final protein concentration was 12.3 mg/ml and FCCP (2 μM) was added as indicated: (a) no additions; (b) 1 mM β-OH butyrate added; (c) 1 mM β-OH butyrate, 500 μM ouabain and oligomycin (5 μg/ml) added. The numbers in parentheses are the respective R.C.I. values.

particular substrate employed. Ouabain and oligomycin were added to diminish ATP turnover and indeed enhanced respiratory control by inhibiting the basal respiration rate (trace c). Respiration in all instances was virtually abolished by conventional respiratory inhibitors. These results indicated that mitochondria *in situ* possessed a degree of respiratory control comparable to that of isolated mitochondria (8).

Exogenous Ca^{+2} decreased R.C.I. values however its influence was complex. Ca^{+2} stimulated the basal oxygen uptake rate and also inhibited the uncoupled respiratory rate. The complexity of Ca^{+2} effects precluded interpretation of its influences in terms of mitochondrial transport of this ion at least by the technique of respiratory control release. R.C.I. values of untreated cells were typically decreased by over 40% by 500 μM Ca^{+2}. Ca^{+2} could have inhibited respiration by decreasing the substrate permeability of

the plasma membrane (9) although this requires additional studies. In the experiments described here, Ca^{+2} was omitted from the perfusion medium and all assays were performed in the presence of EGTA to avoid the above complications.

Cellular Cation Levels

The Na^+ and K^+ contents of isolated cells are summarized in Table I.

Table I

K^+ and Na^+ Levels of Fresh and Treated Cells

Treatment	Cation Level (μeq/mg protein) K^+	Na^+
None (Fresh Cells)	.104	.123
Choline-Cl	.032	.029
KCl	.272	.035
NaCl	.046	.327

Table I. Treated cells were dialyzed at 0-4° C for 2 hrs against 100 volumes of one of the following media: 10 mM Tris-Cl, 2.5 mM Tris-PO_4 (pH 7.2) plus 140 mM Choline-Cl, KCl or NaCl. The values are averages for two cell preparations.

To distinguish their effects, the intracellular levels of Na^+ and K^+ were manipulated by dialysis at 0-4° C. Dialysis against Na^+ or K^+ media readily increased levels of these cations whereas the same treatment with choline medium led to their depletion. Dialysis had no apparent deleterious effects upon mitochondria (e.g. uncoupling) since the R.C.I. values for choline and K^+ treated cells were slightly higher than fresh cells (Table II). There was little difference in the respiratory control of K^+ depleted (choline treated) cells and K^+ treated cells. In contrast, passive loading of cells with Na^+ significantly decreased respiratory control. This effect of Na^+

Table II

Respiratory Control: Influence of Inhibitors and Intracellular Cations

Treatment	R.C.I.* Control	+ Ouabain, Oligomycin
None	3.9 ± .1 (13)	6.7 ± 1.2 (14)
Choline-Cl	4.6 ± .5 (2)	7.4 ± 0.8 (4)
KCl	4.6 ± .8 (5)	8.7 ± 1.2 (6)
NaCl	2.9 ± .5 (4)	5.1 ± 0.1 (4)

*R.C.I. = Respiration Rate + FCCP/Basal Rate

Table II. Cells were treated as described in the legend to Table I. The reaction conditions were the same as employed in trace (c) of Fig. 1. The final protein concentrations ranged from 5-10 mg/ml and 2 μM FCCP was used throughout. Average R.C.I. values, ± S.E., were computed from the number of separate preparations tested as indicated in parentheses.

depended upon the substrate as is described in a later section.

Apparent K^+ Permeability of Mitochondria

Cells were depleted as described in Table I by dialysis against isotonic choline. Release of respiratory control by FCCP (Fig. 2, trace a) was compared to that obtained with valinomycin, a K^+ conducting "ionophore" (10). The respiration of K^+ depleted cells was not effected by valinomycin (trace b) unless supplemented with K^+ (not shown). K^+ treated cells were substantially uncoupled by the ionophore (trace c). It is noteworthy that basal respiratory rates were the same for cells either depleted of K^+ or equilibrated with 140 mM KCl since their K^+ contents differed 9-fold (cf. Table I). Furthermore, maximal uncoupling of K^+ depleted cells by valinomycin occurred with 20 mM exogenous KCl whereas basal respiration was not effected by KCl at concentrations as high as 140 mM. Ouabain and oligomycin served as controls in these experiments to prevent stimulation of respiration by ADP liberated

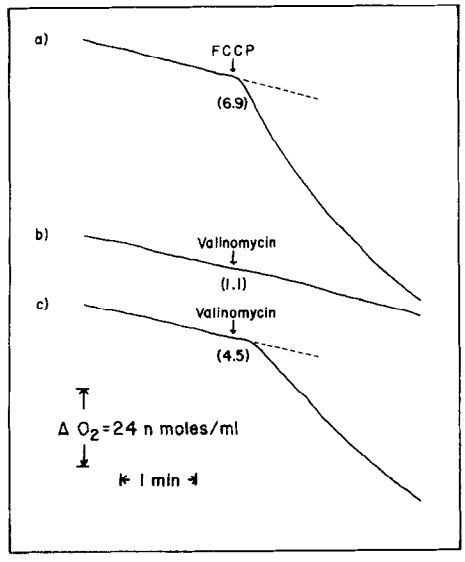

Fig. 2. Traces (a) and (b) were obtained with choline treated cells and trace (c) with K^+ cells. The reaction conditions were identical to those indicated for trace (c) of Fig. 1 and R.C.I. values are also included.

---

by K^+-dependent ATP hydrolysis (either associated with cyclic flow of K^+ across the plasma membrane or possible stimulation of mitochondrial ATPase activity). The valinomycin requirement for uncoupling by K^+ provided strong evidence that mitochondria *in situ* possessed no detectable K^+ conductance.

Influence of Na^+ Upon Cell Respiration

The R.C.I. values for Na^+ treated cells were lower than those of the other cells examined (Table II). Several substrates were tested to determine whether this was a general influence of Na^+ upon respiration. Differences in uncoupled rates of respiration and R.C.I. values proved to depend upon the substrate employed. Results obtained with cells treated with physiological K^+ levels in comparison to cells equilibrated with isotonic NaCl are summarized in Table III. In the presence of β-OH butyrate, the oxygen uptake rates were approximately the same for K^+ and Na^+ treated cells. The respiration of Na^+ treated cells was significantly slower with pyruvate, malate and particularly succinate.

Table III

Comparison of Uncoupled Respiration Rates of Isolated Liver Cells Treated with K^+ or Na^+

Substrate	O_2 Uptake Rate (n gm atoms O/min·mg protein) K^+ Cells	Na^+ Cells	Δ(%)
β-OH Butyrate	9.0	7.6	16
Pyruvate	8.0	5.6	30
Malate	8.5	4.6	46
Succinate	14.	6.2	56

Table III. Cells were treated with either K^+ or Na^+ as outlined in Table I and the conditions were the same as in the experiments of Table II except 1 mM final concentrations of the indicated substrates were added.

Lower uncoupled respiratory rates in Na^+ treated cells could reflect electrically neutral mitochondrial Na^+ for H^+ exchange (11,12). Such an exchange could inhibit respiration by competing with oxidizable substrate accumulation for the mitochondrial transmembrane pH gradient (11). Na^+/H^+ exchange per se does not explain the apparent substrate selectivities indicated by the results of Table III.

DISCUSSION

Good agreement between the degree of respiratory control release by uncoupling agent of liver cells and isolated mitochondria has been demonstrated. Since uncoupling and proton conductance can be related quantitatively (13), it appears that mitochondria *in situ* possess the same low H^+ conduc-

tance as *in vitro* (14). Similarly, it has been shown that K^+ ions do not uncouple hepatocytes except in the presence of valinomycin which strongly suggests the natural K^+ conductance of mitochondria is also very low. As indicated previously, the apparent inhibition of substrate oxidation by Na^+ is consistent with the operation of electrically neutral mitochondrial cation/H^+ exchange *in situ* although Na^+ may also influence respiration by other means. It should be noted that the present studies have not excluded the existence of a Na^+ conductance of mitochondria *in situ* although this seems unlikely. In view of these results, additional similarities between mitochondrial cation permeabilities *in vitro* and *in situ* are expected.

Since liver mitochondria are capable of rapid translocation of Ca^{+2} ions (15), the most probable intracellular permeant cation is Ca^{+2}. Thus Ca^{+2} would be expected to determine the distribution of anionic metabolites between mitochondrial and cytoplasmic compartments. This would provide one indirect mode of regulation of extra-mitochondrial processes such as glycolysis and gluconeogenesis. The present studies will be extended to include elucidation of possible direct effects of Ca^{+2} (16) as well as Na^+ and K^+ upon glucose metabolism.

REFERENCES

1. Garrison, J. C., and Haynes, R. C. (1973) J. Biol. Chem. 248, 5333.
2. Cornell, N. W., Lund, P., Hems, R., and Krebs, H. A. (1973) Biochem. J. 134, 671.
3. Klingenberg, M. (1970) FEBS Letters 6, 145.
4. Henderson, P. (1971) Ann. Rev. Microbiol. 25, 294.
5. Ingebretsen, W. R., and Wagle, S. R. (1972) Biochem. Biophys. Res. Commun. 47, 403.
6. Layne, E. (1952) Methods in Enzymol. 3, 450.
7. Chance, B., and Williams, G. R. (1955) J. Biol. Chem. 217, 383.
8. Johnson, D., and Lardy, H. (1967) Methods in Enzymol. 10, 94.
9. Manery, J. F. (1966) Fed. Proceed. 25, 1804.
10. Moore, C., and Pressman, B. C. (1964) Biochem. Biophys. Res. Commun. 15, 562.
11. Douglas, M., Dubinsky, W., and Cockrell, R. (1973) 9th International Congress of Biochemistry, Stockholm, Sweden, p. 243.
12. Douglas, M. G., and Cockrell, R. S. (1973) J. Biol. Chem., in press.
13. Skulachev, V. P., Sharat, H. H., and Lieberman, E. H. (1967) Nature 216, 718.
14. Mitchell, P., and Moyle, J. (1967) Biochem. J. 104, 588.
15. Lehninger, A. L., Carafoli, E., and Rossi, C. (1967) Adv. Enzymol. 29, 259.
16. Bygrave, F. L. (1967) Nature 214, 667.