FUNCTIONAL STATE OF CULTURED CHICK EMBRYO CELL MITOCHONDRIA INVESTIGATED IN SITU

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SUMMARY

The cell membrane of cultured chick embryo cells is permeable to Ca^{2+} after treatment with mannitol. Ca^{2+} uptake by mannitol-treated cells can be attributed to the mitochondria. Ca^{2+}/O quotients and acceptor control ratios of such cells and isolated mitochondria are identical.

Digitonin exerts two time-dependent effects on mannitol-treated cells: it increases the maximum extent of Ca^{2+} uptake by 5 - 20 percent. It also partially uncouples respiration from Ca^{2+} accumulation.

It is suggested that mannitol treatment of cultured cells provides an easy way to study Ca^{2+} uptake by mitochondria in situ.

INTRODUCTION

The role of Ca^{2+} in regulation of cell metabolism is well established (for a review see 1). The concentration of this ion in the cytoplasm is kept low by an outwardly directed, ATP driven Ca^{2+} pump located in the cell membrane, and by respiration dependent accumulation by the mitochondria (2 - 5). Studies on Ca^{2+} uptake by whole cells have been difficult to interpret because the integrity of the cell membrane was only insufficiently considered (6 - 8). It was shown only recently that the relative impermeability of the cell membrane for Ca^{2+} appears to play an important role in reactions of intact cells to this ion (9).

The present report shows that CEC*, grown in monolayer culture, exhibit identical ACR** and Ca^{2+}/O quotients to their isolated mitochondria when harvested from the culture flasks in a buffer based on mannitol. Respiration coupled to Ca^{2+} transport is observed only in the presence of added succinate, phosphate and rotenone. Digitonin only slightly increases the extent of Ca^{2+} uptake and a concentration of 0.15 mg/ml or more releases accumulated Ca^{2+} from the mitochondria in situ.

Abbreviations: * = CEC: Chick embryo cells
** = ACR: Acceptor control ratios

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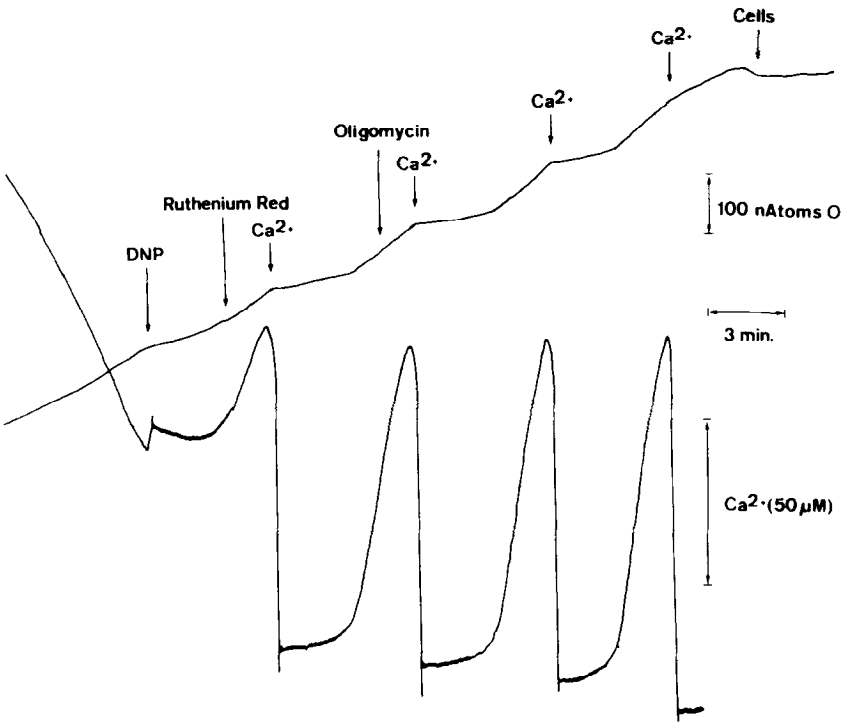

Figure 1: Ca^{2+} uptake by CEC
CEC (protein concentration 3 mg/ml) were incubated at 25° C in the following buffer:
0.25 M mannitol, 10 mM KCl, 5 mM potassium-phosphate, pH 7.2, 1.5 μM rotenone, 5 mM succinate. Total volume was 2.15 ml. The upper curve represents changes of O_2, the lower curve those of Ca^{2+} concentration in the medium.
Ca^{2+} was added as indicated, each time 240 n g-ions of Ca^{2+}. This was followed by stimulation of respiration and disappearance of Ca^{2+} from the medium. Oligomycin (1.74 μg/ml) had no effect on respiratory rate and Ca^{2+} uptake. Ruthenium red (4 μM) inhibited Ca^{2+} stimulated respiration and Ca^{2+} uptake. DNP (43 μM) uncoupled respiration and led to an increase of Ca^{2+} concentration in the medium.

MATERIALS AND METHODS

CEC were prepared from 11 days old white Leghorn embryos by treatment with 0.125 % (w/v) trypsin (Gibco, Grand Island, N.Y. USA), resuspended in 100 ml/flask Tissue Culture Medium 199 (Serva, Heidelberg, Germany) containing 5 % fetal calf serum (Gibco) and seeded out in a Roux bottle (surface 215 cm^2) at a cell density of 10^6/ml. From 48 hours old monolayers, the culture medium was decanted and the cells were washed with 20 ml of mitochondria isolation medium/culture bottle. Isolation medium was composed of 0.27 M mannitol, 0.05 % (w/v) bovine serum albumin (Armour Pharmaceutical Company, Eastbourne, GB), 10 mM Tris-HCl, pH 7.3. When mitochondria were isolated to study oxidative phosphorylation, 0.1 mM EDTA was included in the isolation medium. Cells were

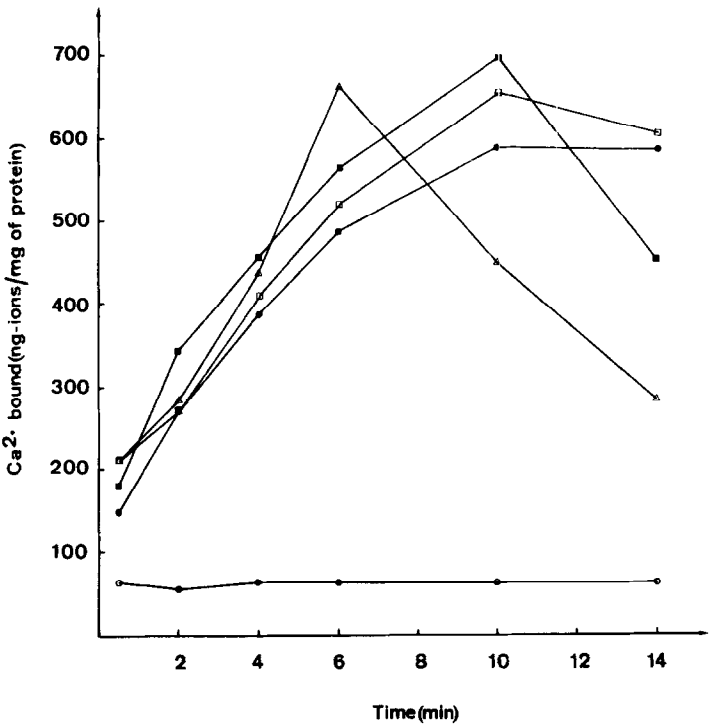

Figure 2: Ca^{2+} uptake by intact CEC: Effect of digitonin

$^{45}Ca^{2+}$ uptake by CEC was measured using a rapid Millipore filtration technique. Incubation medium was as described in the legend to fig. 1. $CaCl_2$ was 1 mM (approx. 300 cpm/n g-ion Ca^{2+}).

Incubation was started by adding CEC (0.8 mg protein).

Samples (20 µl volume) were taken at the times indicated and rapidly filtered through 0.45 µm wet Millipore filters. Filters were rinsed with 0.5 ml of ice cold incubation medium without radioactive Ca^{2+}, dried and counted in a Packard liquid scintillation spectrometer Mod. 3375.

● no digitonin
O no digitonin, 3 µg/ml antimycin A
□ 0.066 mg/ml digitonin
■ 0.15 mg/ml digitonin
△ 0.46 mg/ml digitonin

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then gently detached from the supporting glass in 15 ml of isolation medium and transferred to centrifugation tubes. The Roux bottle was rinsed with 10 ml of the same medium. The rinse was added to the first 15 ml and mixed well. After centrifugation at 600 x g for 7 minutes, the supernatant was decanted and the cell pellet resuspended in either a few drops of medium for assay of in situ mitochondria, or in 30 ml of freshly added medium for subsequent iso-

Table 1: Comparison of Ca^{2+} uptake by intact CEC and by isolated CEC mitochondria.

		RCI	Ca^{2+}/O	Respiratory rate in state 3 (ng atoms O . mg^{-1} . min^{-1})
Cell batch I	Intact cells	4.53 + 0.75	3.48 ± 0.07	27.5 ± 1.27
	Mitochondria	4.57 ± 0.31	3.45 ± 0.12	109.4 ± 4.26
Cell batch 2	Intact cells	3.87 ± 0.34	3.65 ± 0.12	17.7 ± 2.19
	Mitochondria	3.63 ± 0.21	3.5 ± 0.12	72.9 ± 1.67

CEC and mitochondria isolated from the same CEC batch were incubated in the buffer described in the legend to fig. 1. Protein concentration of mitochondria was 0.8 mg/ml and of CEC 1.2 mg/ml, respectively. The volume of the incubation vessel was 1.0 ml. Respiration was stimulated repeatedly by addition of 1.25 n moles of $CaCl_2$. Results are expressed as mean ± SD of 4 successive cycles.

lation of mitochondria. The cells of the latter suspension were broken up in a tight-fitting Dounce homogenizer (11 strokes) and the homogenate was centrifuged at 700 x g for 7 minutes. The resulting supernatant was further centrifuged at 10'000 x g for 10 minutes and the pellet was used for studies with isolated mitochondria. Protein concentrations were determined by the biuret method (10). Oxidative phosphorylation and Ca^{2+} uptake were studied at 25° C in the following assay medium: 0.25 M mannitol, 10 mM KCl, 5 mM potassium-phosphate, pH 7.2. Oxygen concentration of the assay medium was determined using sonicated mitochondria and NADH, and was 485 ng atoms O/ml. Changes of oxygen were measured with a Clark-type electrode (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio, USA) and traces plotted with a W+W recorder 600 (Kontron, Zurich, Switzerland). In some experiments, Ca^{2+} movements in the assay medium were followed simultaneously with the oxygen concentration. The Calcium electrode, Mod. 93-20, was supplied by Orion Research, Cambridge, MA, USA).

ACR, ADP/O and Ca^{2+}/O quotients were calculated as described (11). $^{45}Ca^{2+}$ uptake was measured by a rapid Millipore filtration technique. For details see legend to fig. 2.

Antimycin A and oligomycin were supplied by Calbiochem (Lucerne, Switzerland). Ruthenium red was a gift from Dr. K. Schwerzmann (Eidgenössische Technische Hochschule, Zurich).

RESULTS AND DISCUSSION

Fig.1 shows an experiment in which Ca^{2+} was added to CEC in the presence of succinate, phosphate and rotenone. Changes of Ca^{2+} and oxygen concentrations

Table 2: Oxidative phosphorylation of isolated chick embryo fibroblast mitochondria.

Substrate	ACR	ADP/O	Respiratory rate in state 3 (ng atoms O . mg^{-1} . min^{-1})
Malate-Pyruvate	5.93 ± 0.43	2.99 ± 0.17	55.39 ± 5.65
β-Hydroxybutyrate	3.55 ± 0.17	2.66 ± 0.13	50.93 ± 6.31
α-Ketoglutarate	4.82 ± 0.48	2.84 ± 0.10	70.1 ± 8.41
Succinate	2.88 ± 0.23	1.7 ± 0.03	72.48 ± 2.03

Mitochondria (1 mg/ml of protein) were incubated at 25° C in a vessel containing 1.0 ml of incubation medium (0.25 M mannitol, 10 mM KCl, 5 mM potassium phosphate, pH 7.2. Concentration of succinate, α-ketoglutarate and β-hydroxybutyrate was 5 mM, malate-pyruvate 1 mM - 5 mM, respectively.

4 cycles of ATP synthesis were started by addition of 120 n moles of ADP. Results are expressed as mean ± SD obtained with three batches of mitochondria isolated separately from the same batch of cell cultures.

were recorded simultaneously. Addition of Ca^{2+} is followed by a transient stimulation of respiration. During this period, Ca^{2+} disappears from the medium. Oligomycin does not inhibit Ca^{2+} uptake while ruthenium red and antimycin A (shown in fig. 2) do so. Uncoupling of respiration by dinitrophenol is followed by a rapid release of accumulated Ca^{2+} into the medium. These observations suggest that in CEC only the mitochondria are able to actively accumulate significant amounts of Ca^{2+}.

Functional parameters of in situ and isolated mitochondria are compared in table 1. Both RCI and Ca^{2+}/O are in the same range when compared within identical cell batches. Respiratory control of in situ mitochondria was observed only when succinate, in the presence of rotenone, was used as the substrate. This is in line with oberservations reported for Ehrlich ascites tumor cells (9).

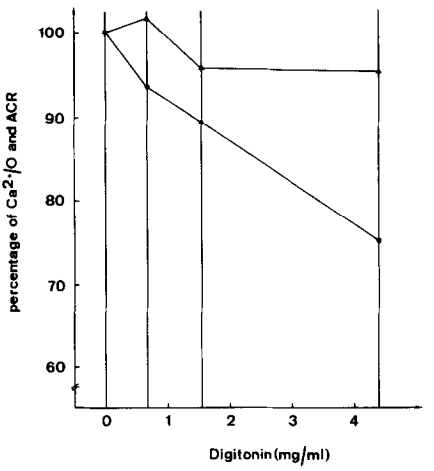

Figure 3: Ca^{2+} uptake by intact CEC:
Effect of digitonin on Ca^{2+}/O quotient and ACR. Intact fibroblasts were incubated as described in table 2. Results are expressed in percent of values obtained in the absence of digitonin.

▲ Ca^{2+}/O quotient
● ACR

Since isolated mitochondria are also able to carry out oxidative phosphorylation (table 2), one may conclude that the isolation technique used in these studies results in a functionally intact mitochondrial preparation. From this and from the similarity of ACR and Ca^{2+}/O between in situ and isolated mitochondria, we conclude that these two parameters, measured in situ, reflect the true functional state of mitochondria.

For an identical reaction of in situ and isolated mitochondria to Ca^{2+}, a high permeability of the cell membrane is necessary. Therefore, we analyzed whether the permeability of the cell membrane could be further increased by digitonin which has been used for this purpose in hepatocytes (7).

$^{45}Ca^{2+}$ uptake of whole cells was measured using a rapid Millipore filtration technique. The maximum extent of $^{45}Ca^{2+}$ uptake varied with the cell batch, and ranged from 300 - 700 n g-ions of Ca^{2+}/mg of cellular protein.

Fig. 2 shows that the maximum extent of $^{45}Ca^{2+}$ uptake was higher by 5 - 20 percent when digitonin was present in the incubation medium. At the highest concentration of digitonin (0.46 mg/ml), maximum uptake was reached faster and the accumulated $^{45}Ca^{2+}$ was rapidly released.

The complex effect of digitonin could be explained as follows:

a) Digitonin facilitates the access of Ca^{2+} to the mitochondria by increasing the permeability of the cell membrane and/or other cellular membranes. It may also allow Ca^{2+} to come in contact with mitochondria which are not accessible in mannitol-treated cells.

b) It partially uncouples respiration from Ca^{2+} accumulation.
The latter point is supported by experiments in which Ca^{2+}/O and ACR of whole CEC were determined in the presence of digitonin (fig. 3). Increasing concentrations of this drug result in a gradual loss of respiratory control. Uncoupling became more pronounced with prolonged incubation (not shown). This would explain why the maximum uptake of $^{45}Ca^{2+}$ was not lower at 0.46 mg/ml of digitonin, and why the mitochondria were unable to retain the accumulated $^{45}Ca^{2+}$. A time dependency of the effect of digitonin on mitochondria has also been shown by Zuurendonk and Tager (13).

Our experiments clearly indicate that it is possible to investigate the functional state of mitochondria in "intact" CEC during Ca^{2+} accumulation. The term "intact" deserves some explanation. The cell membrane of mannitol-treated CEC is permeable for Ca^{2+} and is therefore, functionally, not intact. $^{45}Ca^{2+}$ uptake of CEC harvested and assayed in a NaCl medium (composition given in 9) was only a few percent of that observed in the mannitol system (not shown). Similar low uptake was observed when $^{45}Ca^{2+}$ uptake of CEC was measured on culture dishes in the presence of cell culture medium. (Unpublished observation). The latter method, especially, avoids any mechanical damage to the cell and the composition of the cell culture medium allows the cell membrane to maintain the normal ion and substrate gradients to the extracellular space. A combination of experiments of this type, together with the one reported in this paper, would allow net Ca^{2+} regulation by intact CEC to be investigated, and to differentiate effects at the cell membrane from those which are due to the mitochondria.

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REFERENCES

1. Carafoli, E., Malmström, K., Sigel, E., and Crompton, M. (1976) Clin. Endocrinol., 5, suppl., 49s - 59s

2. Lehninger, A.L. (1964) The Mitochondrion, pp. 175 - 178, W.A. Benjamin, New York

3. Lehninger, A.L. (1970) Biochem. J., 119, 129 - 138

4. Bygrave, F.L. (1966) Biochem. J., 101, 279 - 281

5. Rose, B., and Loewenstein, W.R. (1975) Nature, 254, 250 - 252

6. Borle, A.B. (1973) Fed. Proc. Fed. Am. Soc. Exp. Biol., 32, 1944 - 1950

7. Levinson, C., and Blumenson, L.E. (1970) J. Cell. Physiol., 75, 231 - 240

8. Cittadini, A., Scarpa, A., and Chance, B. (1973) Biochim. Biophys. Acta, 291, 246 - 259

9. Landry, Y., and Lehninger, A.L. (1976) Biochem. J., 158, 427 - 438

10. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) J. Biol. Chem., 177, 751 - 766

11. Estabrook, R.W. (1956) Methods in Enzymology, 10, pp. 41 - 48, Academic Press, New York

12. Dubinsky, W.P., and Cockrell, R.S. (1975) FEBS Letters, 59, 39 - 43

13. Zuurendonk, P.F., and Tager, J.M. (1974) Biochim. Biophys. Acta, 333, 393 - 399