KINETIC EVIDENCE FOR Ca^{2+} UPTAKE BY INTACT EHRLICH ASCITES TUMOR CELLS

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1. Introduction

The importance of Ca^{2+} in controlling the structure, adhesiveness and permeability of cells [1] and in regulating cellular activity and metabolism [2] has been established. For this reason, several studies on the effect of Ca^{2+} on tumor tissue have been performed and yet little is known about the transport and distribution of Ca^{2+} in tumor cells. The present investigation analyzes the ability of tumor cells to transport Ca^{2+} under appropriate metabolic conditions. The ascites tumor cells has been used as a model for the present study. This cell exists in suspension *in vivo* and can be isolated without contaminants and with a minimum of disturbance of cell characteristics.

Thomason and Schoffield [3] first observed a Ca^{2+} exchange by ascites tumor cells *in vivo*. On the other hand, experiments with $^{45}Ca^{2+}$ led Bygrave [4] to the conclusion that ascites cells are unable to take up Ca^{2+} *in vitro*. In further studies, Levinson and Blumenson [5] described different compartments for the exchangeable $^{45}Ca^{2+}$ and concluded that ascites tumor cells do not have an energy dependent accumulation of Ca^{2+}. However, these studies performed with $^{45}Ca^{2+}$ required long periods for sampling and separation of the cells from the medium and are therefore inadequate for measuring the rapid kinetics of ion movements under varying metabolic conditions.

The use of murexide as Ca^{2+} indicator has recently made possible the spectrophotometric measurement of Ca^{2+} movement and binding in sarcoplasmic reticulum [6], intact muscle [7] and isolated mitochondria [8]. We present here evidence that murexide can be successfully used as a tool for meausirng kinetics of Ca^{2+} uptake in intact ascites tumor cells. In this way, we were able to demonstrate that Ca^{2+} penetrates intact ascites tumor cells *in vitro* and, depending on the metabolic state, these cells display an energy linked Ca^{2+} accumulation.

2. Materials and methods

Ascites tumor cells (Ehrlich-Lettre' hyperdiploid) were harvested 6–8 days after inoculation in ICR albino mice. The cells were washed once and resuspended at a concentration of about 70–80 mg d. wt/ml in a medium containing 0.225 M mannitol–0.075 M sucrose–10 mM sodium morpholinopropanesulphonate (MOPS), pH 7.4. This medium was used in all the experiments to avoid possible interference from other cations on Ca^{2+} movements. Qualitatively similar results were obtained with a saline-phosphate medium [9] and in both media the cells display similar characteristics with respect to glycolitic activity, respiration and permeability to substrates.

Ca^{2+} movements were followed either polarographically using a Ca^{2+} sensitive electrode (Orion Res. Inc., Cambridge, Mass.) [10] or spectrophotometrically by monitoring the formation and the disappearance of the Ca-murexide complex with a dual wavelength spectrophotometer at 540–507 nm [8]. It has been demonstrated that murexide neither binds to nor moves through the mitochondrial membrane [8]. By centrifugation and spectrophotometric assay of murexide in the supernatant, we were able to verify that murexide was extracellular to ascites cells in all our experimental conditions. Cytochrome *b* redox changes were measured by the dual wavelength tech-

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nique [11] in the Soret region at 430–410 nm. All the experiments were carried out at room temperature.

The 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was kindly supplied by Dr. R.B. Beechey of Shell Research, Sittingbourne (England). Murexide (ammonium purpurate) and Lanthanum were purchased from K and K Chemicals, Plainview (New York).

3. Experimental

Ca^{2+} movement was followed in intact ascites tumor cells by both polarographic and spectrophotometric techniques as shown in fig. 1. The bottom trace reproduces a recording of a Ca^{2+} sensitive electrode. The addition of $CaCl_2$ in a stirred vessel containing cells incubated in the presence of rotenone, produces an upward deflection corresponding to an increase of the $[Ca^{2+}]$ present in the medium. The trace thereafter remains linear, indicating that Ca^{2+} is not disappearing from the medium. Upon addition of succinate, the slow downward deflection indicates the disappearance of Ca^{2+} from the medium to the cells. The amount of Ca^{2+} accumulated under these conditions is approximatively 75% of that added and is complete in about 4 min. The following addition of the uncoupling agent TTFB gives rise to an opposite deflection indicating that Ca^{2+} is again increasing in the external medium. After 4 min, it is possible to see that all the Ca^{2+} accumulated by the cells has been released. In a parallel experiment under identical conditions, the same phenomena were followed by the murexide technique (upper trace). In this case, the addition of $CaCl_2$ produces a rapid downward deflection of the trace which corresponds to a decrease in absorbance at 540 nm due to the formation of the Ca-murexide complex. The increase in absorbance after addition of succinate and the decrease after addition of TTFB indicate that Ca^{2+} is taken up by the cells and released to the medium respectively. A comparison of the upper and lower traces shows the identity of the two measurements obtained by different techniques.

Fig. 2A and B compares the Ca^{2+} uptake by cells under different metabolic conditions. The figure shows that the Ca^{2+} uptake is, in both rate and extent, dependent on the substrate or the inhibitor with which the cells were incubated. In the absence of added substrate, the cells are able to accumulate about 50 μM Ca^{2+} in 3 min. This uptake is followed by a complete release which can be explained as the

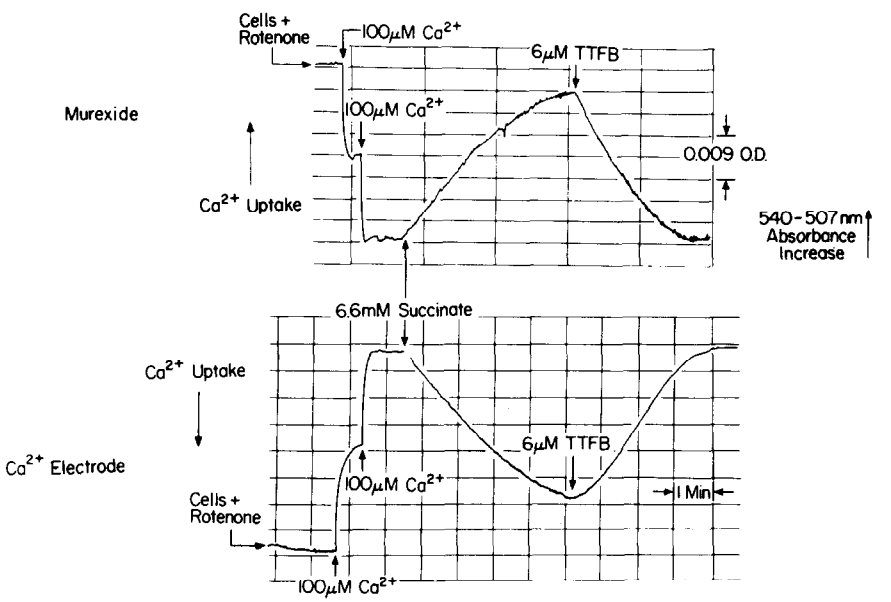

Fig. 1. Ca^{2+} uptake by intact ascites tumor cells monitored spectrophotometrically (murexide) and polarographically (Ca^{2+} sensitive electrode). The cells were suspended at a concentration of 18 mg d. wt/ml and incubated with 6.7 μM rotenone. For spectrophotometric measurements the medium was supplemented with 50 μM murexide.

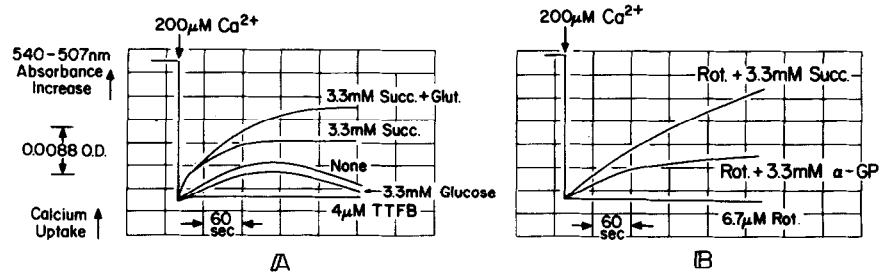

Fig. 2A and B. Ca^{2+} uptake by intact ascites tumor cells under different metabolic conditions. The cells were 10 mg d. wt/ml and murexide 50 μM.

exhaustion of endogenous substrates. Incubation of the cells with glucose gives rise to a similar but diminished biphasic Ca^{2+} response. The effect of glucose on Ca^{2+} accumulation has also been studied under anaerobic conditions and no Ca^{2+} uptake could be detected (data not shown). Preincubation of the cells with either succinate or succinate plus glutamate initiates a large Ca^{2+} uptake which is complete in 5 min. These responses are not observed if respiration is uncoupled by TTFB.

Fig. 2B shows that the cells are also unable to accumulate Ca^{2+} if incubated in the presence of rotenone. However, in the presence of rotenone, succinate or α-glycerophosphate restores the ability of the cells to accumulate Ca^{2+}. The slower rate of Ca^{2+} uptake in the presence of α-glycerophosphate is explained by the low rate of oxidation of this substrate by this strain of ascites cells [12]. On the other hand, the enhancement of Ca^{2+} uptake by succinate in the presence of rotenone, with respect to succinate alone, is probably due to the block of reversed electron transfer [13] and the channeling of more energy into Ca^{2+} accumulation.

Since our experiments suggested a mitochondrial involvement in the cellular Ca^{2+} transport, the role of mitochondria during Ca^{2+} uptake by ascites tumor cells has been further characterized. It is well known that Ca^{2+} uptake by isolated mitochondria induces changes in the redox state of the respiratory chain components [14]. We studied similar mitochondrial changes in intact cells upon addition of Ca^{2+} to the extracellular medium. This is shown in fig. 3 where

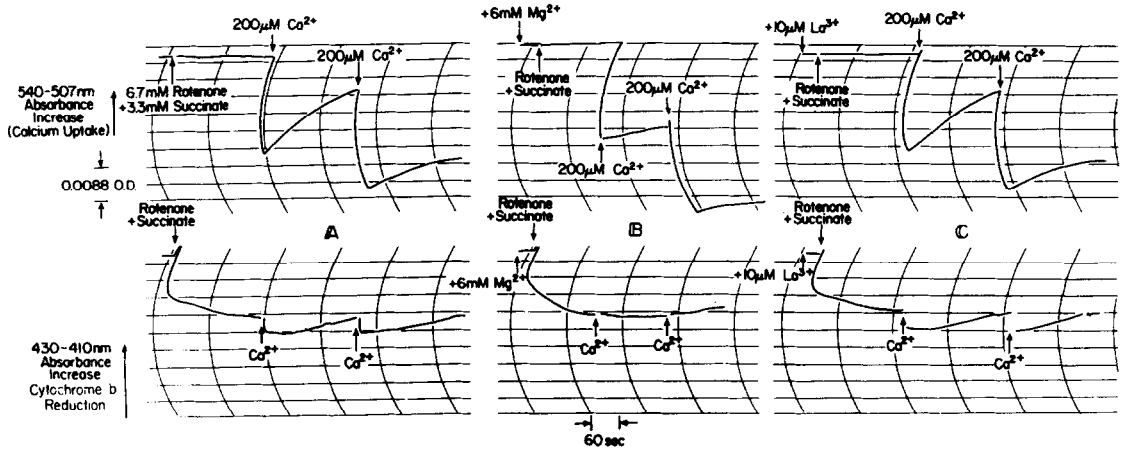

Fig. 3A, B and C. Parallel measurements of Ca^{2+} uptake and cyt. *b* redox state in intact ascites tumor cells. For the Ca^{2+} measurements the medium was supplemented with 50 μM murexide. The cells were 9.2 mg d. wt/ml.

the cellular Ca^{2+} uptake was measured simultaneously with the redox changes of mitochondria cyt. *b*. Fig. 3A shows that the addition of rotenone and succinate to the cells does not affect the murexide trace. However, the cyt. *b* trace shows a downward deflection, corresponding to an oxidation. The subsequent addition of 200 μM $CaCl_2$ produces the same biphasic response of murexide as in fig. 2, indicating that the Ca^{2+} added is progressively taken up by the cells. A concomitant cyt. *b* oxidation, followed by a slow reduction occurs. A second addition of $CaCl_2$ stimulates Ca^{2+} uptake and produces a new cyclic cyt. *b* oxidation. Fig. 3B shows the same experiment under conditions where Ca^{2+} penetration into the cells was blocked. This was obtained in presence of Mg^{2+} which we found to be an inhibitor of the cellular Ca^{2+} accumulation but not Ca^{2+} transport by mitochondria isolated from the same cells [15]. In the presence of 6 mM $MgCl_2$, Ca^{2+} transport of the whole cell is 90% inhibited and there is no detectable cyclic cyt. *b* oxidation. Fig. 3C shows the effect of La^{3+}, a specific inhibitor of mitochondrial Ca^{2+} transport [16]. La^{3+} at a concentration of 3 μM is able to block by 90% the uptake of the Ca^{2+} by mitochondria isolated from ascites tumor cells [15]. However, fig. 3C shows that a larger amount of $LaCl_3$ (10 μM) does not affect either Ca^{2+} accumulation by cells or cyt. *b* redox state. This lack of inhibition can be explained in different ways, such as the insensitivity of the cellular transport mechanism to La^{3+}, or the impermeability of the cell to La^{3+} or the precipitation of La^{3+} inside the cell.

4. Discussion

The correspondence of the measurements obtained with a Ca^{2+} electrode and with murexide, together with the evidence of the extracellular distribution of this dye, made possible the use of murexide as indicator of the extracellular concentration of Ca^{2+} in ascites tumor cells. The fast response time, the minor interference by other components, and the higher sensitivity render murexide a valuable tool for kinetic measurements of Ca^{2+} accumulation by these cells.

All the results presented here clearly show that isolated ascites tumor cells are able to take up Ca^{2+}. This accumulation occurs in the presence of endogenous substrates and is enhanced by added mitochondrial substrates. The inhibition by rotenone and by TTFB also indicates that this process depends on energy-coupled respiration. However, in the presence of glucose, which supports Ca^{2+} accumulation by other cellular systems [17], we were unable to detect any Ca^{2+} uptake.

The involvement of mitochondria in the transport by whole cells has also been confirmed by the experiments shown in fig. 3, where we have shown that the mitochondrial cyt. *b* responds to changes of the extracellular Ca^{2+} concentrations with cyclic oxidations. Although mitochondrial uptake could account for all the cellular Ca^{2+} accumulation, another role involving the mitochondria cannot be excluded. The inhibitory effect of Mg^{2+} and the lack of inhibition by La^{3+} on the cellular Ca^{2+} transport only indicate the presence of additional control mechanisms, different from those regulating the Ca^{2+} uptake by isolated mitochondria.

To conclude, our evidence clearly demonstrates an energy-dependent transport of Ca^{2+} by intact ascites tumor cells. Earlier studies in which Ca^{2+} has been reported either not to be accumulated [4] or to be exchanged by an energy independent process [5] should be reconsidered.

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