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CALCIUM EXCHANGE IN EHRLICH MOUSE ASCITES TUMOR CELLS

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SUMMARY

1. The mean Ca^{2+} content of Ehrlich mouse ascites tumor cells was 0.131 μmole per 10^7 cells.

2. Ca^{2+} addition to the cellular environment was unnecessary for the maintenance of high internal K^+ content and low internal Na^+ content. The Ca^{2+} content of the cell was also independent of the external Ca^{2+} concentration.

3. Low concentrations of $HClO_4$ (1–10%) extracted 30% of the cellular Ca^{2+} while the remaining 70% was tenaciously bound to the $HClO_4$-insoluble residue.

4. The cell Ca^{2+} which was extractable with low concentrations of $HClO_4$ exchanged 3 times faster than the Ca^{2+} which remained bound.

5. The results are interpreted as evidence for Ca^{2+} compartmentation within the tumor cell.

INTRODUCTION

Although there is wide interest in calcium metabolism of malignant tissues[1,2], information regarding the manner in which tumor cells regulate this ion is not abundant. Previous studies have been primarily concerned with the influence of calcium ions on the permeability of other ions and molecules, and relatively little is known about the penetration of the calcium ion itself[3].

Since the cell membrane may play an important role in the regulation of calcium metabolism in the tumor cell, the content of this ion and its flux across the cell membrane were measured.

METHODS

Experiments were performed with Ehrlich–Lettré ascites tumor cells that were maintained in Ha/ICR swiss mice by weekly transplantation. Tumor-bearing animals with growths between 7 and 12 days were used. Methods for the preparation of cell suspensions have been described previously[4]. 1×10^7 cells corresponded to 24 mg wet wt. or 4.7 mg dry wt.

Since distilled water contained varying amounts of Ca^{2+}, all reagents and solutions were prepared in Baxter water (Baxter Laboratories, Morton Grove, Ill.).

Depending upon the experiment, the tumor cell suspension was divided into 2 or more equal aliquots and washed in Ringer's solution of varying Ca^{2+} concentration (0–12 mM). The final solutions were pH 7.4. Usually 10–20 ml of cell suspension (2×10^7–4×10^7 cells/ml) were added to each 250-ml flask and allowed to equilibrate 60 min under 100% O_2 at 22–26°.

In experiments where ^{45}Ca was used, 2 μC (carrier-free; New England Nuclear, Boston, Mass.) per ml cell suspension were added at time zero and sampling begun. Duplicate aliquots of 1 or 2 ml were centrifuged for 35 sec at 2000 $\times$ g and stopped by mechanical braking. The supernatant was decanted and replaced with ice-cold isosmotic choline chloride solution buffered to pH 7.2 with 0.003 M Tris–HCl. The cells were resuspended and washed twice. Less than 4 min elapsed from the initial to final centrifugation. To one sample, 10 ml of Baxter water were added; and after 60 min Na^+ and K^+ were analysed by flame photometry[5]. The second sample of cells were suspended in a small volume of Baxter water, and quantitatively transferred to crucibles which were then slowly heated to 500° and maintained at that temperature for 16 h. The ash was dissolved in 5.0 ml of 2.5% HCl (v/v) containing 15 mM lanthanum. 1 ml was added to 10 ml of BRAY's scintillation mixture[6], and the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. The remaining 4 ml were used for the determination of Ca^{2+} by atomic absorption spectroscopy.

In later experiments parallel samples of the washed, packed cells were extracted with $HClO_4$ before ashing. Usually 2 ml of cold 7% $HClO_4$ (v/v) were added to the cell sample and after thorough mixing in the cold the clear $HClO_4$ extract was transferred to crucibles. The $HClO_4$-insoluble material was then washed twice with cold choline chloride and added to other crucibles. These samples were then ashed and treated as described.

RESULTS

Ca^{2+} content

In order to test the atomic absorption procedure for the determination of Ca^{2+} in the tumor cell, experiments were performed in which known amounts of Ca^{2+} were added to ashed samples of the same population of cells. Table I shows the result of a typical experiment. In 3 experiments performed recoveries and reproducibilities were generally good. The mean Ca^{2+} content from 22 different populations of cells was 0.131 ± 0.016 (S.E.) μmole/10^7 cells with a range of 0.015 to 0.210 μmole/10^7 cells. No positive correlation existed between the age of the tumor and Ca^{2+} content. In 3 separate experiments the ascitic fluid was found to contain 3.4 mM Ca^{2+} (mean value). 'Free' Ca^{2+} was estimated by diluting samples of the ascitic fluid and performing the analysis on unashed samples. The mean, free Ca^{2+} content was 2.3 mM.

Since the ascitic fluid contained appreciable Ca^{2+}, experiments were performed to test whether external Ca^{2+} was essential for maintaining a high internal K^+ and a low internal Na^+ content. In the absence of added Ca^{2+} to the Ringer solution the cell content of Na^+, K^+ and Ca^{2+} did not vary appreciably from that found at 12 mM Ca^{2+}.

TABLE I

RECOVERY OF CHEMICAL Ca^{2+}

To samples of ashed cells Ca^{2+} was added, and then analyzed by atomic absorption spectroscopy. The per cent recovery was calculated from μmoles Ca^{2+} added/μmoles Ca^{2+} recovered. Result of a typical experiment.

Sample	*Ca^{2+} added (μmoles)*	*Ca^{2+} recovered (μmoles)*	*Recovery (%)*
Ashed cells	0.0	0.651	—
	0.5	1.130	98.5
	0.9	1.466	94.5
	1.3	2.010	103.2
	2.0	2.831	106

Ca^{2+} exchange

To measure Ca^{2+} exchange, ^{45}Ca was added to the cell suspension equilibrated with 2.5 mM Ca^{2+}. The increase in radioactivity was then measured in sequential samples. The spec. activity of the 'cell Ca^{2+}' was calculated and expressed as counts/min per μmole Ca^{2+} while rel. spec. activity equalled spec. activity (cell, at time t)/spec. activity (cell, at equilibrium). Fig. 1 (lower portion) shows the result of a typical uptake experiment. Note that when these data were treated with the kinetics of a 2-compartment closed system[7,8] at least 2 processes became apparent (upper portion).

To determine whether Ca^{2+} compartmentation could explain the observed kinetic behavior, experiments were designed to fractionate the total Ca^{2+} pool. These results are shown in Table II. Note that from 1% to 10% cold $HClO_4$ extracted a fairly constant amount of the total cell Ca^{2+}. This fraction was equal to about 30% of the intracellular pool. At 20% $HClO_4$, however, more than 60% was extracted. Thus at relatively low $HClO_4$ concentrations a uniform fraction of Ca^{2+} could be separated from the total pool, but as the $HClO_4$ concentration increased more bound Ca^{2+} was liberated.

Since intracellular Ca^{2+} could be separated into $HClO_4$-soluble and $HClO_4$-insoluble, these 2 fractions might represent the 2 components of Ca^{2+} exchange seen in Fig. 1. Experiments were performed in which the Ca^{2+} content and uptake of ^{45}Ca

TABLE II

$HClO_4$ EXTRACTION OF CELL Ca^{2+}

Increasing concentrations of cold $HClO_4$ were added to packed, washed cells, and the resulting $HClO_4$ extract and residue analyzed by atomic absorption spectroscopy for Ca^{2+}. Mean values from 2 experiments.

$HClO_4$ (%, v/v)	*μmoles/10^7 cells*		
	Extractable Ca^{2+}	*Non-extractable Ca^{2+}*	*Total*
1	0.055	0.126	0.181
7	0.060	0.114	0.174
10	0.069	0.120	0.189
20	0.101	0.065	0.167

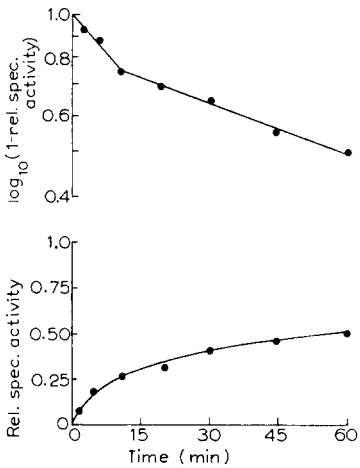

Fig. 1. The kinetics of cell Ca^{2+} exchange. Upper curve represents a plot of log (1 − rel. spec. activity) *versus* time. Lower curve shows increase in rel. spec. activity with time. At t_0, ^{45}Ca was added to the cell environment (2.5 mM Ca^{2+}), and the increase in spec. activity of cell Ca^{2+} was measured over a 60-min period.

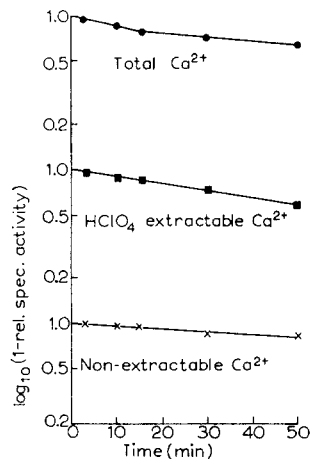

Fig. 2. The kinetics of cell Ca^{2+} exchange. log (1 − rel. spec. activity) *versus* time for the total Ca^{2+} pool, 7% $HClO_4$-extractable Ca^{2+}, and non-extractable Ca^{2+} is shown. At t_0, ^{45}Ca was added to the medium (2.5 mM Ca^{2+}), and the increase in spec. activity of the total, $HClO_4$-extractable, and non-extractable Ca^{2+} was measured over a 50-min period.

were measured simultaneously in the total Ca^{2+} compartment and in the extractable (7% $HClO_4$) and non-extractable fractions. Fig. 2 shows the results of this type of experiment. The top curve indicates that Ca^{2+} exchange can be represented by 2 different rates. The middle and lower curves which represent exchange with $HClO_4$-extractable and non-extractable Ca^{2+} respectively, follow a single exponential. The efflux rate coefficients were 0.851/h for the extractable Ca^{2+} and 0.311/h for the non-extractable Ca^{2+}.

Since the cells were in the steady state as determined by the constancy of the internal Ca^{2+} content, influx equals efflux and equalled the product of the efflux rate coefficient times the compartment size. The exchange flux measured in 4 separate experiments were 0.041±0.006 (S.E.) μmole/10^7 cells per h for the fast or extractable compartment and 0.084±0.015 (S.E.) for the slow or non-extractable compartment.

DISCUSSION

The results reported here show that the mean Ca^{2+} content of Ehrlich ascites tumor cells was 0.131 μmole/10^7 cells or 5 μmoles/g wet wt. While this value is higher than that reported by THOMASON AND SCHOFIELD[9] both studies showed that the Ca^{2+} content of the tumor cell was quite variable. It was not possible to relate variation in Ca^{2+} content to the age of the tumor. Analyses of the Na^+ and K^+ content of the cells also showed that the internal content of these ions was independent of the intracellular Ca^{2+} content within the defined limits.

The observation that external Ca^{2+} was unnecessary for the maintenance of high internal K^+ and low internal Na^+ confirms the findings of HEMPLING[10] and of

AULL AND HEMPLING[11]. These authors showed that at room temperature tumor cells maintained high internal K^+ and low internal Na^+ content, and in addition actively transported these ions in the absence of added external Ca^{2+}. Furthermore, HEMPLING[12] has shown that these cells when incubated in Ca^{2+}-free Ringer solution at 25° behaved as osmometers which suggested that permeability to NaCl was not increased in the absence of external Ca^{2+}. MORILL, KABACH AND ROBBINS[13], on the other hand, reported that the internal K^+ and Na^+ content of the tumor cell at 37° was sensitive to external Ca^{2+}. In their experiments the absence of Ca^{2+} led to a loss of K^+ and to a gain of Na^+.

Although it was observed that there was no net penetration of Ca^{2+} into the cell, it was of interest to determine whether cell Ca^{2+} exchanged with that of the environment. THOMASON AND SCHOFIELD[9,14] found that *in vivo* all of the cellular Ca^{2+} exchanged with Ca^{2+} of the ascitic fluid. However, these authors stated that at room temperature, under *in vitro* conditions, no exchange was demonstrable. This puzzling observation may be related to the fact that during their *in vitro* experiments the cells were not washed free of exogenous ^{45}Ca and that this radioactivity was sufficiently great to mask incorporated radioactivity. In a recent paper BYGRAVE[15] also reported the non-exchangeability of cellular Ca^{2+}. Since the decrease in environmental ^{45}Ca was used to assess Ca^{2+} exchange and a relatively low cell concentration was used, the fraction of ^{45}Ca appearing in the cells even at isotopic equilibrium would have been less than 10% of the initial radioactivity.

It is evident from the data in Fig. 1 that ^{45}Ca when added to the environment exchanged with cellular Ca^{2+}. It was observed, however, that this exchange deviated from the kinetic behavior predicted by a two-compartment closed system. The deviation was most likely due to the existence of at least 2-cell Ca^{2+} compartments which turned over at different rates. This interpretation is derived from the fact that a fraction of the cellular Ca^{2+} could be extracted by $HClO_4$ (Table II). For example, when a low concentration of $HClO_4$ was added to the cells about 30% of the total Ca^{2+} was recovered in the $HClO_4$ extract while 70% remained tenaciously bound to the $HClO_4$-insoluble residue. However, as the $HClO_4$ concentration was increased, progressively more Ca^{2+} was removed from the cell. This suggested that a fraction of the Ca^{2+} was tightly bound to structural components on or within the cell, and that increasing concentrations of $HClO_4$ were necessary to free this Ca^{2+}. Similarly, HARRIS[16] in his studies of Ca^{2+} turnover in frog muscle concluded that Ca^{2+} was principally in a bound form, but the strength of binding was variable.

When the $HClO_4$-extractable and non-extractable Ca^{2+} fractions were treated independently with the kinetics of a two-compartment closed system (Fig. 2) only one efflux rate coefficient was found for each. This showed that the kinetic behavior seen in the total Ca^{2+} compartment could be resolved into 2-separate processes occurring within the Ca^{2+} pool.

The loosely bound or $HClO_4$-extractable Ca^{2+} turned over about 3 times faster than the slower or non-extractable Ca^{2+}. The data in Fig. 2 suggests the extractable Ca^{2+} is synonymous with the fast exchanging component while the non-extractable or tightly bound Ca^{2+} represents the slower exchanging fraction. Thus it is likely that most of the Ca^{2+} ions associated with the tumor cell are bound to cell organelles, including the cell periphery but not necessarily to the same degree.

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