INTRACELLULAR CALCIUM AND MAGNESIUM CONTENT AND AEROBIC LACTATE PRODUCTION IN INTACT EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

The high rate of aerobic glycolysis and the low 'Pasteur effect' are the most consistent biochemical features of tumour cell energy metabolism ([1], reviewed [2]) but their causes and meaning are still under discussion [3–6].

Among various hypotheses proposed to explain the high aerobic glycolysis of cancer cells one, postulating a profound alteration of cell calcium metabolism, seems very attractive as it could explain some other biological features relevant to neoplasia (viz., uncontrolled growth, invasivity ([3], reviewed [7]). This hypothesis implies the existence in cancer cells of a decreased cytosolic Ca^{2+} concentration resulting from both a low plasma membrane permeability to Ca^{2+} and a high Ca^{2+} accumulating capacity of tumour mitochondria. The following alteration of the Ca^{2+}/Mg^{2+} ratio would then be responsible for the increased activity of some key glycolytic enzymes [3].

Following our results on Ca^{2+} extrusion by intact Ehrlich ascites tumour cells (ATC) [8,9] we now report data on the relationship between calcium content and rate of aerobic glycolysis in ATC. For this purpose we utilized, as experimental model, intact ATC suspended in isosmotic, Tris-buffered, mannitol–sucrose solution, since in this medium ATC show an unusual low rate of aerobic glycolysis; in addition we took advantage of the antibiotic ionophore A23187, which has been shown to strongly increase ATC calcium content [8,9].

The results obtained led us to the conclusion that in intact ATC the intracellular calcium concentration, and the size of its exchangeable pool, do play a major role in the regulation of aerobic glycolysis and that the calcium effect is strictly related to depletion of cellular magnesium.

2. Material and methods

Ehrlich ATC, hyperdiploid strain, were grown by weekly intraperitoneal transplantation, on Swiss albino mice. The cells harvested after 7–8 days were washed, centrifuged and resuspended in the following medium: 0.225 M mannitol, 0.075 M sucrose, 0.0013 M $CaCl_2$, 0.001 M $MgSO_4$ and 0.010 M Tris–HCl (pH 7.4) (MST).

For intracellular calcium and magnesium determination the cells, after incubation under the suitable metabolic conditions, were quickly separated from the suspension medium by layering 1 ml suspension upon 4 ml 0.3 M salt-free sucrose. The tubes were rapidly centrifuged at 3200 rev./min for 75 s in a bench centrifuge. The cell sediement was dried in an oven at 100°C for $\geqslant$ 4 h, then extracted with 0.1 N HNO_3. Calcium and magnesium were determined in the acid extracts by atomic absorption spectrophotometry, with 1% $LaCl_3$ added to both samples and standards [8]. This procedure gives reliable estimates of intracellular cations with negligible contamination from the suspending medium [8,10].

Oxygen consumption was determined by Warburg manometric technique over 1 h incubation at 38°C and was linear over the 60 min incubation.

Lactate, glucose-6-phosphate (G6P) and fructose-1,6-diphosphate (FDP) were enzymatically determined at 366 nm with an Eppendorf photometer [11].

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The size of the intracellular exchangeable pool of Ca^{2+} was determined by the use of $^{45}Ca^{2+}$. In these experiments ~0.5 μCi/ml of carrier-free tracer were added to MST solution containing the usual amount of cold cation (1.3 mM). After the suitable incubation the cells were separated from suspending medium and extracted as above. $^{45}Ca^{2+}$ was determined, both in the suspension medium and cell extracts by liquid scintillation counting with the appropriate correction for background and efficiency.

3. Results

Figure 1 shows the effect of increasing A23187 concentration upon ATC calcium and magnesium content and aerobic lactate production. The calcium curve shows a typical pattern [9] with a small initial decrease at 1 μM ionophore concentration and a continuous increase thereafter. The most interesting result of this experiment is that, in parallel with the increase of calcium content, the aerobic lactate production increases ~5-fold, while magnesium content decreases by ~60 mmol/kg cell protein.

A23187's effect on lactate production and ATC calcium content is prevented by the addition of 3 mM EGTA (ethylene glycol di-(aminoethyl) tetracetic acid) to the incubation medium. Table 1 shows that, in the presence of EGTA, A23187 provokes a decrease of cellular calcium, which is drained out of the cell by the combined action of both the chelating agent and the increased plasma membrane permeability due to the ionophore. Under these conditions both lactate production and magnesium level remain unchanged.

The decrease of Mg^{2+} by itself cannot be considered responsible for the increased lactate accumulation brought about by A23187, as proved by the following experiment with EDTA (ethylene di-amino tetracetic acid). In the presence of this compound magnesium level is lowered by A23187 to about half the value obtained in the presence of EGTA, while lactate production is not enhanced (table 1).

In the presence of excess Mg^{2+} (5 mM), A23187 is again able to stimulate ATC lactate production, although to a lower extent if compared with control conditions. With 5 mM magnesium cell calcium level is lower than in the controls. In all these conditions A23187 provokes Mg^{2+} loss (Δ from −23 to −53

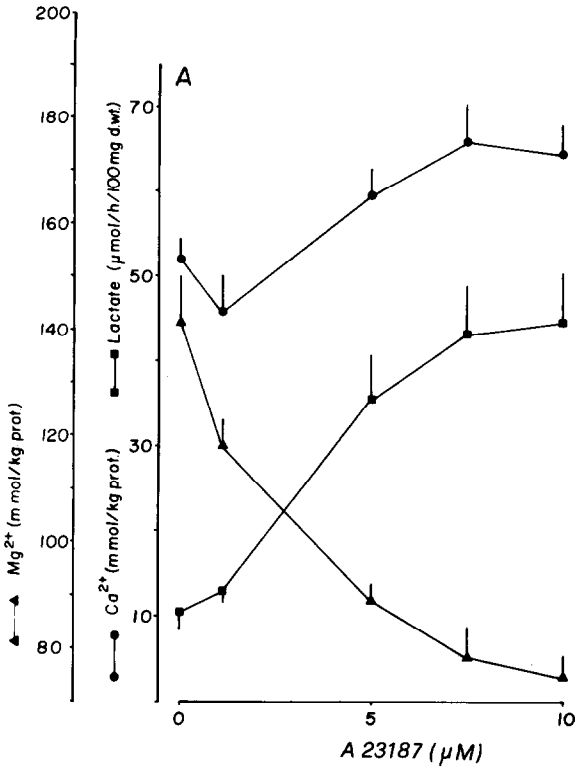

Fig.1. Effect of the ionophore A23187 upon calcium and magnesium content and aerobic lactate production in ATC. The cells preincubated at 0–4°C for 120 min were transferred to shaking flasks and incubated 60 min under O_2 at 38°C with 20 mM glucose. Cell calcium and magnesium content and lactate production were determined at the end of the incubation as in section 2. ATC calcium content (●——●), after a small decrease at 1 μM A23187, increases at higher concentrations. ATC magnesium content (▲——▲) sharply decreases. Aerobic lactate production (■——■) is strongly stimulated by increasing A23187 concentrations. Results are given as mean ± SEM of 10 expt analyzed in triplicate.

mmol/kg cell prot.) but induces an increase of lactate accumulation only when calcium enters the cell with a $\Delta Mg^{2+}/\Delta Ca^{2+}$ ratio of about−2 (table 1).

On the other hand an increase of ATC calcium without loss of magnesium does not influence the aerobic lactate production. This is illustrated by the results plotted in fig.2. In these experiments ATC were incubated at 38°C after preincubation at 0–4°C up to 300 min. During the cold incubation ATC take up calcium that is then extruded at 38°C by an

Table 1

Effect of the ionophore A23187 on total cell calcium and magnesium content and aerobic lactate production in intact ATC suspended in isotonic, buffered mannitol–sucrose solution, in the presence of EGTA, EDTA and excess Mg^{2+}

	A23187 (10 μM)	Lactate (μmol .100 mg dry $wt^{-1}.h^{-1}$)	Δ	Calcium (mmol .kg $prot.^{-1}$)	Δ	Magnesium (mmol .kg $prot.^{-1}$)	Δ	$\frac{\Delta Mg^{2+}}{\Delta Ca^{2+}}$
Control	–	7.6 ± 0.5 (29)	+31.3	87.5 ± 12.5 (10)	+23	131.7 ± 5.1 (9)	−53	−2.3
	+	38.9 ± 3.7 (30)		110.9 ± 6.5 (10)		79.2 ± 3.0 (9)		
EGTA 3 mM	–	11.9 ± 2.3 (8)	+ 2.2	67.5 ± 8.9 (8)	−32	109.6 ± 11.8 (8)	−23	+0.7
	+	14.1 ± 1.5 (8)[a]		35.1 ± 4.6 (8)		86.2 ± 5.8 (8)		
EDTA 3 mM	–	14.0 ± 2.8 (8)	+ 1.0	55.5 ± 9.4 (8)	−33	88.9 ± 9.5 (7)	−42	+1.3
	+	15.0 ± 3.5 (8)[a]		23.0 ± 4.4 (7)		46.5 ± 6.9 (7)		
Mg^{2+} 5 mM	–	9.4 ± 1.6 (10)	+12.3	35.2 ± 2.3 (18)	+13	136.9 ± 12.3 (18)	−29	−2.2
	+	21.7 ± 2.0 (14)		48.6 ± 2.6 (26)		108.2 ± 9.4 (26)		

[a] Not statistically different from its control without ionophore; all the other differences are statistically significant with $p < 0.0005$

Glucose, 20 mM, throughout. Mean values ± SEM are given with the no. expt in parentheses

energy-dependent mechanism [8]. Nevertheless, the longer the preincubation at 0–4°C the higher the level of residual intracellular calcium after the incubation at 38°C. Figure 2 also shows that under these conditions neither the level of magnesium nor lactate accumulation undergo appreciable changes.

Table 2 summarizes data showing that after prolonged incubation at 0–4°C ATC are still responsive to A23187 treatment with regard to Ca^{2+} increase, Mg^{2+} depletion and lactate production.

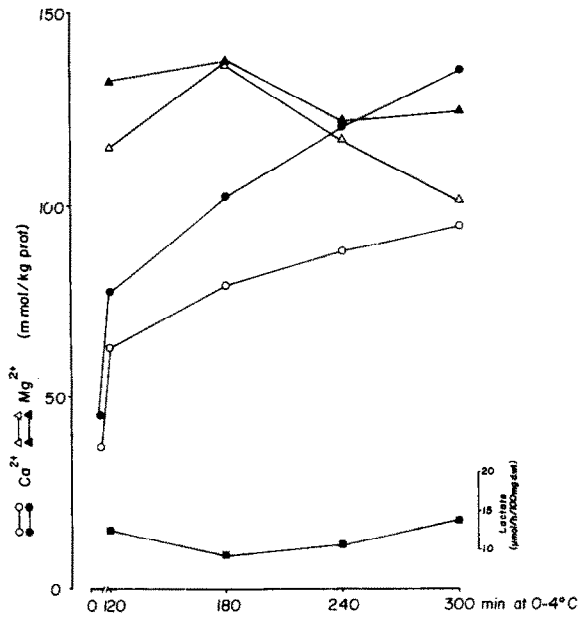

Table 3 summarizes the rate of oxygen consumption of ATC incubated under different conditions. A23187 inhibited ATC respiration by 65% in the presence of glucose and by ~80% in the presence of pyruvate. Both EGTA and EDTA inhibit by ~15% ATC respiration with glucose, but do not suppress the effect of the ionophore (about −60%). With succinate as substrate there is only a small, not significant, inhibition of respiration in the presence of A23187.

The observation that, in the presence of EGTA and EDTA, A23187 still provokes substantial reduction of ATC oxygen uptake while there is no increase of lactate accumulation (table 1), indicates that inhibition of respiration by A23187 cannot be considered responsible for the stimulation of lactate

Fig.2. Effect of prolonged incubation at 0–4°C on calcium and magnesium content and aerobic lactate production in intact ATC. ATC (~3 mg dry wt/ml) were incubated at 0–4°C up to 5 h. At time intervals, cell suspension aliquots were transferred to O_2 gassed shaking flasks and incubated at 38°C for 60 min; at the end of the 38°C incubation ATC calcium and magnesium content and lactate production were measured. The substantial cell calcium increase brought about by the prolonged cold incubation does not reflect changes either of cell magnesium or aerobic lactate production. (▲) Mg^{2+} at 0–4°C; (△) Mg^{2+} at 38°C; (●) Ca^{2+} at 0–4°C; (○) Ca^{2+} at 38°C; (■) aerobic lactate production at 38°C. 20 mM glucose throughout. Data plotted from a typical experiment repeated 3 times with similar results.

Table 2
A23187 effect on calcium, magnesium and aerobic lactate production in Ehrlich ATC incubated at 0–4°C for a prolonged time

0–4°C	38°C	A23187 (10 μM)	Calcium (mmol .kg cell prot.$^{-1}$)	Magnesium	Lactate (μmol .100 mg dry wt^{-1} .h^{-1})
180 min	–	–	100.5[a]	97.7	–
180 min	60 min	–	50.1	96.3	4.0
180 min	60 min	+	79.0	61.0	30.2
240 min	–	–	130.0	110.3	–
240 min	60 min	–	61.0	114.3	3.3
240 min	60 min	+	78.1	58.7	36.4
300 min	–	–	120.4	135.2	–
300 min	60 min	–	63.0	116.1	3.4
300 min	60 min	+	75.1	73.5	34.8

[a] The values are the mean of 4 different observations

Cells incubated in the presence of 20 mM glucose

Table 3
Effect of the antibiotic ionophore A23187 upon oxygen uptake of Ehrlich ATC incubated in the presence of various substrates and EGTA or EDTA

Additions	A23187 (10 μM)	Q_{O_2} (μl .h^{-1} .mg dry wt^{-1})
Glucose (20 mM)	–	6.4 ± 0.2 (4)[a]
Glucose (20 mM)	+	2.2 ± 0.2 (4)
Glucose (20 mM) + EGTA (3 mM)	–	5.5 ± 0.3 (4)
Glucose (20 mM) + EGTA (3 mM)	+	2.5 ± 0.2 (4)
Glucose (20 mM) + EDTA (3 mM)	–	5.4 ± 0.3 (4)
Glucose (20 mM) + EDTA (3 mM)	+	1.9 ± 0.4 (4)
Pyruvate (10 mM)	–	8.8 ± 0.6 (5)
Pyruvate (10 mM)	+	2.6 ± 0.4 (5)
Succinate (10 mM)	–	13.7 ± 0.7 (4)
Succinate (10 mM)	+	11.8 ± 0.2 (4)

[a] SEM (no. observations)

Table 4
Effect of the ionophore A23187 on glucose-6-phosphate (G-6-P) and fructose-1,6-diphosphate (FDP) levels in Ehrlich ATC incubated 60 min under aerobiosis with 20 mM glucose as substrate

	G-6-P (μmol .100 mg dry wt^{-1})	FDP (nmol .100 mg dry wt^{-1})
Control	2.0 ± 0.05 (8)	10.4 ± 3.5 (5)
A23187 (10 μM)	0.9 ± 0.1 (10)	24.3 ± 4.0 (9)

Table 5
Effect of the antibiotic ionophore A23187 (10 μM) on the size of Ca^{2+} exchangeable pool in intact Ehrlich ATC

	Total intracellular calcium (nmol .kg prot.$^{-1}$)	$^{45}Ca^{2+}$ cell/medium spec. act. %	Size of the exchangeable pool (mmol .kg prot.$^{-1}$)
Control	52.0 ± 2.0 (6)	27.1 ± 3.4 (8)	15.0
A23187	71.4 ± 4.2 (6)	97.2 ± 3.8 (12)	69.4

Ehrlich ATC, suspended in isosmotic, buffered, mannitol–sucrose solution containing 1.3 mM $CaCl_2$, were incubated at 38°C in the presence of 10 μM A23187 and 20 mM glucose with oxygen for 30 min, time necessary to reach steady state, as proved in control experiments. At that time carrier-free $^{45}Ca^{2+}$ (about 0.5 μCi/ml) was added to the suspension, and after additional 30 min, both intracellular total and $^{45}Ca^{2+}$ were determined as in section 2

accumulation in absence of chelators.

The following experiments were performed in order to study whether an increase in cellular Ca^{2+} might affect the PFK reaction in vivo. As a measure of PFK activity glucose 6-phosphate and fructose-1,6-diphosphate levels were determined [12]. As shown in table 4, in the presence of A23187 glucose-6-phosphate is reduced to half the control value, while FDP is doubled, corresponding to a 4-fold increase of the G6P/FDP ratio. Thus it appears that PFK is activated in ATC incubated in the presence of A23187. The low level of FDP in our experiments may be explained to be due to a strong inhibition of PFK caused by the absence of both K^+ and P_i [12] in the incubation medium.

The effect of the ionophore A23187 on intracellular calcium exchange properties and on the size of exchangeable calcium pool in intact ATC is reported in table 5. It can be seen that the addition of 10 μM A23187, besides the increase of intracellular total calcium already described in fig.1, elicits a deep modification of Ca^{2+} exchangeability. The $^{45}Ca^{2+}$ cell/medium specific activity % ratio increases from 27–97 in the ionophore-treated cells. From the knowledge of the above data it is possible to calculate that A23187 provokes a 4-fold increase in size of the intracellular exchangeable calcium pool (last column of table 5).

4. Discussion

For a long time calcium has been considered responsible for the fine regulation of cell metabolic activity. Several hypotheses suggest stimulation or inhibition by Ca^{2+} of some key enzymes, either mitochondrial or cytosolic [13–15]. In particular, in cancer cells the alteration of cytosolic Ca^{2+} concentration has been proposed to influence glycolytic enzymes (e.g., pyruvate kinase) [3,13].

Here we present evidence to show how modifications of intracellular calcium content and its distribution among intracellular pools may elicit large changes in lactate accumulation and O_2 consumption by ATC.

The experimental model we used in this work is particularly suitable for this purpose, since ATC suspended in mannitol–sucrose medium show a low rate of aerobic lactate production, while respiration is comparable to that in conventional medium (unpublished observations).

Modifications of the content and binding state of ATC calcium were obtained by the use of the antibiotic ionophore A23187, that equilibrates calcium across biological membranes [8,16,17] many of which (e.g., mitochondria and endoplasmic reticulum) are involved in the regulation of cytosolic calcium concentration [15,18]. That ATC mitochondria do accumulate Ca^{2+} is a well known fact [4].

In our experiments A23187 substantially increased intracellular calcium concentration and modified its binding state (fig.1, tables 1, 5). The increase can be attributed to the cytosolic compartment, since, in the presence of the ionophore, those compartments that take up Ca^{2+} actively are collapsed and likely in equilibrium with the surrounding cytosol [20,21].

Simultaneously with the calcium increase there is a loss of magnesium. This cation, one of the most stable in the cell [19], is linked to important activities located both at the mitochondrial and the cytosolic level. Mitochondrial ATPase requires magnesium, while Mg-ATP exerts a strong inhibition of cytosolic enzymes, the most important of which in this context is PFK. However the loss of magnesium brought about by A23187 appears not sufficient to explain the metabolic changes observed in intact ATC. Thus an increase in lactate production was observed only when the depletion of magnesium was associated with an accumulation of calcium. In this case lactate accumulation is even proportional to the amount of calcium that has entered the cell (table 1). In the presence of EGTA or EDTA cell magnesium is strongly reduced but lactate accumulation is not stimulated since Ca^{2+} is drained from the cell.

The inhibition of oxygen uptake of the tumour cells observed upon addition of the ionophore may be attributed to several causes acting together. First it should be noted that the inhibition is seen only with glucose and pyruvate as substrates but not with succinate. Many mitochondrial enzymes require Ca^{2+} for their activity and one of these is pyruvate dehydrogenase phosphate phosphatase [15], the interconvertible system of which has been demonstrated also in ATC [22]. This enzyme might be inhibited by the calcium loss that probably takes place from mitochondrial compartment with the ionophore. The inhibition of respiration could be also due to other effects of A23187 on mitochondria as pointed out by many authors (reviewed [20]), but at the moment we cannot explain why in intact ATC A23187 induces inhibition of O_2 consumption and not uncoupling, as shown in isolated mitochondria [21]. Probably the situation of mitochondria in vivo is different from that in vitro.

In ionophore-treated ATC inhibition of respiration is not the cause of the increased lactate accumulation because, in the presence of EGTA and EDTA, the addition of A23187 provokes inhibition of respiration (table 3) but no increase of lactate accumulation, since cell Ca^{2+} remains at a low level (table 1).

Substantial increase of lactate accumulation occurs only if an increase of cell calcium is associated with a decrease of cell magnesium. An increase of cell Ca^{2+} not associated with magnesium loss (as that obtained in the conditions of fig.2) is ineffective in inducing the metabolic changes. Our results show that besides the absolute levels of Ca^{2+}, also its distribution among intracellular pools is an important feature in determining ATC metabolic characteristics. In particular, PFK reaction in vivo is stimulated not only by a de-inhibition due to magnesium loss [12] but also by calcium increase. This of course does not rule out the possibility that Ca^{2+} also regulates the activity of other glycolytic enzymes.

Our findings do not fit with the hypothesis that implies a decreased cytosolic Ca^{2+} level to explain the high aerobic glycolysis of cancer cells [3]. However, at the moment it is difficult to interprete our data against that hypothesis because of the peculiar conditions of our experiments. They were performed, in fact, in a medium free of monovalent cations, physiologically relevant; nevertheless, the results obtained constitute good evidence for the role that Ca^{2+}, together with Mg^{2+}, may play in the regulation of ATC glycolysis.

Further experiments are required to better define the behaviour of the entire glycolytic pathway in the conditions of our experiments, and to assess the role of the described phenomenon in neoplastic cell glycolysis behaviour.

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