Biochimica et Biophysica Acta, 645 (1981) 177–182
Elsevier/North-Holland Biomedical Press

BBA 79314

LACK OF EFFECT OF THE Ca^{2+} IONOPHORE A23187 ON TUMOUR CELLS

A. CITTADINI, D. BOSSI, A.M. DANI, G. CALVIELLO, F. WOLF and T. TERRANOVA

Institute of General Pathology, Catholic University, School of Medicine, Roma (Italy)

(Received December 31st, 1980)

Key words: Ionophore A23187; Ca^{2+} permeability; (Tumor cell)

The Ca^{2+} ionophore A23187 increases intracellular calcium content in normal thymic cells, while it is without effect on the corresponding neoplastic cell (Ascites thymoma) and on Ehrlich ascites tumour cells. The A23187-induced total cell calcium increase in normal thymocytes takes place both in control and energy-depleted cells, while it is lacking in neoplastic cells. In addition the ionophore stimulates aerobic glycolysis of normal thymocytes, whereas it is ineffective on neoplastic cells. The study of intracellular calcium exchange properties reveals that in normal cells the ionophore A23187 provokes a 60% increase of the exchangeable pool together with a more significant, 4-fold enlargement of the unexchangeable pool. These effects are lacking in cancer cells. The data give rise to interesting considerations concerning the regulation and compartmentalization of calcium in neoplastic cells. The results will be also discussed in relation to the models that predict altered cell calcium metabolism as a cause of cancer cell high aerobic glycolysis and uncontrolled growth.

Introduction

Alteration of cell Ca^{2+} metabolism has been proposed by many authors to be an important pathogenetic step in the development of cancer cell biological and biochemical features [1–3]. Indeed, changes of either net content or movement and compartmentalization of cellular calcium would satisfy several requirements for the explanation of the neoplastic cell's most consistent characteristics, namely high aerobic glycolysis and uncontrolled growth. In fact, the regulation of aerobic glycolysis and cell multiplication is inherently linked to the level and movement of intracellular Ca^{2+} [4–6]. At the present, however, the available information is fragmentary and often contradictory, so that the problem of whether and how cancer cell Ca^{2+} metabolism is altered remains an open question. In an attempt to clarify some aspects of the Ca^{2+}-tumour cell relationship and following our previous investigations on ascites cell Ca^{2+} metabolism [7,8], we have studied the effect of the ionophore A23187 on calcium content and aerobic glycolysis of neoplastic cells.

A23187 is an antibiotic ionophore known to equilibrate Ca^{2+}, as well as other divalent cations with decreasing affinity, across biological membranes [9] both in subcellular organelles (e.g., mitochondria [10] and microsomes [11]) and in whole cell preparations [12–15]. This compound has been recently utilized as a tool for the determination of cytosolic Ca^{2+} buffering activity [16] as well as for the measure and control of ionized Mg^{2+} in intact red blood cells [17].

In this study we utilized, as an experimental model, two strains of ascites tumour cells, one of which, the ascites thymoma, presents the advantage of being directly comparable with normal thymic lymphocytes, so providing valuable information about neoplastic transformation of lymphoid tissue. Furthermore, these isolated cell preparations are particularly useful since they allow direct determination of intracellular ions without contamination from interstitial tissue.

The results obtained show that the ionophore

A23187 significantly increases calcium and decreases magnesium content in normal thymocytes; at the same time a strong stimulation of aerobic glycolysis occurs. By contrast, A23187 fails to induce calcium increase in both strains of tumour cells studied, while it slightly decreases the aerobic lactate production of these cells. The analysis of the Ca^{2+} exchange properties of cells revealed that in normal cells the ionophore provokes strong modification of the intracellular Ca^{2+} pool, while it is ineffective on cancer cells. The lack of A23187 effect upon cancer cells indicates that the inwardly directed Ca^{2+} leak is uncontrolled in these cells as compared to normal cells, irrespective of the presence of the specific Ca^{2+} ionophore.

Material and Methods

Ehrlich ascites tumour cells (hyperdiploid strain) were grown, harvested and washed as usual (see Ref. 7 for technical details). Ascites thymoma cells (kindly supplied by the Institute of General Pathology of the University of Perugia) were grown in BALBc mice by weekly intraperitoneal transplantation and harvested after 7–8 days. Normal thymus cells were obtained from young, healthy rats as described elsewhere [18]. All cell suspensions were washed twice and resuspended in the following medium: 146 mM NaCl, 5.0 mM KCl, 1.0 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 10.0 mM Tris-HCl pH 7.4 (Ringer-Tris). Cell calcium and magnesium content was determined as previously described [19], by atomic absorption spectrophotometry on the acid cell extract (0.1 M HNO_3) in the presence of 1% $LaCl_3$ added to both samples and standards. In the experiments on $^{45}Ca^{2+}$-exchange properties, carrier-free $^{45}Ca^{2+}$ (0.5 μCi/ml) was added to cell suspensions preincubated 30 min with 1.3 mM Ca^{2+} in order to reach steady state. After an additional 30 min the cells were withdrawn and treated as described in Ref. 19. $^{45}Ca^{2+}$ was determined by liquid scintillation counting. Lactate production, O_2 consumption and protein concentration were measured according to Ref. 19. A23187 was kindly supplied by Lilly Res. Labs., Indianapolis, IN, U.S.A.

Results and Discussion

Table I summarizes the data on the effect of the antibiotic ionophore A23187 upon total cell calcium and magnesium content in Ehrlich ascites, thymoma ascites and normal thymus cells. In the presence of A23187 (1.0 μM) the normal thymocyte net calcium content strongly increases by about 200% (from 13.8 to 40.4 mmol/kg protein) while a 20% magnesium loss takes place. By contrast, neither Ehrlich nor thymoma ascites cell calcium content shows significant modification upon addition of A23187 (10.0 μM), while magnesium content decreases by about 30% in the ascites thymoma. It must be noted that the concentration of A23187 effective on normal cells is 10-times lower than that without effect on cancer cells. In the latter, even higher A23187 concentrations (up to 100 μM) are without specific effect (not shown), while in normal thymocytes higher concentrations exhibit an effect similar to that obtained with 1.0 μM A23187.

It could be argued that, in the present experiment conditions, with Na^+ and K^+ largely exceeding Ca^{2+} concentration in the suspension medium, A23187 becomes less available for Ca^{2+} because of its binding to monovalent cations. The results of Table I, however, clearly exclude this evidence for two

TABLE I

EFFECT OF A23187 UPON TOTAL CELL CALCIUM AND MAGNESIUM CONTENT OF TWO STRAINS OF ASCITES TUMOUR CELLS AND ISOLATED NORMAL THYMUS CELLS

Cells incubated 60 min at 38°C under aerobiosis with 20 mM (A, B) and 10 mM (C) glucose

	A23187 (μM)	mmol/kg protein	
		Calcium	Magnesium
(A) Ehrlich ascites	–	21.5 ± 3.9 (6)	58.1 ± 3.3 (6)
tumour cells	10.0	22.9 ± 5.0 (8)	59.0 ± 5.0 (3)
(B) Ascites	–	9.8 ± 1.0 (14)	74.4 ± 1.1 (16)
thymoma cells	10.0	10.2 ± 1.0 (13)	54.2 ± 2.2 (15)
(C) Normal thymus	–	13.8 ± 0.4 (22)	61.6 ± 1.8 (25)
cells	1.0	40.4 ± 1.6 (10)	49.6 ± 1.8 (28)

reasons: (a) one-tenth of the concentration ineffective on cancer cells is, indeed, fully effective on normal cells suspended in the same medium; (b) 10 μM A23187 does decrease significantly Mg^{2+} while it does not increase Ca^{2+}, in the ascites thymoma cells. The lack of effect of A23187 on cancer cell calcium content thus, must be considered specific, rather than due to ionophore deficiency caused by monovalent cations competition.

The net increase as well as the influx of Ca^{2+} into the cell has been shown to bring about aerobic glycolysis stimulation in several cell systems [19,20]. The results of Table II show that A23187 stimulates aerobic lactate accumulation in normal thymic cells by about 200%. The strong glycolysis enhancement is directly dependent on A23187-induced cell calcium increase, since it does not take place when calcium entry is prevented by extracellular Ca^{2+} chelation with excess EGTA (data not reported). Quite different results have been obtained with cancer cells. Table II shows that in neoplastic cells, both Ehrlich and thymoma ascites cells, the aerobic glycolysis rate is not affected by the ionophore. At most, A23187 slightly inhibits cancer cell aerobic glycolysis. A23187 does not affect either Ehrlich or normal thymus cell O_2 uptake, but it inhibits thymoma respiration by 20%. It must be underlined that the high rate of aerobic glycolysis is the most consistent biochemical characteristic of cancer cells. Since the discovery of this, many attempts have been made to understand its pathogenesis; among others, some hypotheses suggest that cell calcium metabolism derangement is the cause of this alteration [2,21,22].

We have shown in a previous paper that Ehrlich ascites tumour cells, suspended in non-physiological, ion-free medium, exhibited a very low rate of aerobic glycolysis and that, in the same experimental conditions, the increase of intracellular calcium provoked a strong stimulation of aerobic lactate accumulation. The effect was ascribed to the consistent increase of the intracellular exchangeable Ca^{2+} pool [19].

The effect of A23187 on Ca^{2+}-exchange properties has been also investigated in Ehrlich and thymoma ascites as well as in normal thymus cells under the present experimental conditions. The ionophore does not elicit any significant effect on Ehrlich and thymoma ascites cell steady-state $^{45}Ca^{2+}$ per cent exchange, while it decreases by about 50% $^{45}Ca^{2+}$ cell/medium specific activity in normal thymocytes. From these data and from cell total calcium content (Table I), the size of intracellular exchangeable and unexchangeable pools can be calculated (second and third column of Table III). It is clear that in cancer cells, both exchangeable and unexchangeable Ca^{2+} pools do not show changes upon ionophore addition. By contrast, A23187 strongly increases the intracellular Ca^{2+} pools in normal thymocytes. The exchangeable pool becomes about 60% larger and the unexchangeable one is conspicuously increased by about 300%.

The results so far reported indicate that the Ca^{2+} ionophore is unable to increase total calcium content

TABLE II

EFFECT OF A23187 UPON AEROBIC LACTATE PRODUCTION AND O_2 UPTAKE BY EHRLICH AND THYMOMA ASCITES CELLS AND NORMAL THYMOCYTES

Cells incubated in Warburg vessels (0.2 ml 20% KOH in the central well) at 38°C with glucose as substrate. O_2 consumption determined at 10-min intervals over a 60-min period. Lactate measured on the acid extract (perchloric acid 7%) enzymatically at 366 nm with an Eppendorf photometer.

	A23187 (μM)	Lactate (μmol/100 mg dry wt. per h)	O_2 uptake (μl/mg dry wt. per h)
(A) Ehrlich ascites tumour cells		39.8 ± 1.1 (4)	6.7 ± 0.2 (4)
	10.0	29.1 ± 0.3 (3)	6.6 ± 0.4 (3)
(B) Ascites thymoma cells	–	16.9 ± 1.2 (8)	5.9 ± 0.3 (7)
	10.0	12.0 ± 1.5 (8)	4.2 ± 0.3 (8)
(C) Normal thymus cells	–	7.2 ± 0.2 (21)	5.6 ± 0.2 (13)
	1.0	19.5 ± 1.3 (6)	5.6 ± 0.2 (8)

TABLE III

EFFECT OF A23187 ON PERCENT EXCHANGE AND SIZE OF INTRACELLULAR EXCHANGEABLE AND UNEXCHANGEABLE CALCIUM POOLS IN INTACT ASCITES TUMOUR AND NORMAL THYMUS CELLS

The cells, suspended in Ringer-Tris containing 1.3 mM $CaCl_2$ and supplemented with glucose, were incubated under O_2 for 30 min in order to reach steady state, as checked in control experiments. Thereafter carrier-free $^{45}Ca^{2+}$ (about 0.5 μCi/ml) was added and after an additional 30 min of incubation cells were separated from suspending medium (see Ref. 19) and their content of tracer was measured.

	A23187 (μM)	$^{45}Ca^{2+}$ cell/medium specific activity (%)	Size of intracellular Ca^{2+} [a] pools (mmol/kg protein)	
			Exchangeable	Unexchangeable
(A) Ehrlich ascites tumour cells	–	38.7 ± 4.6 (12)	8.3	13.2
	10.0	44.7 ± 4.2 (16)	10.2	12.7
(B) Ascites thymoma	–	41.0 ± 2.3 (12)	4.0	5.8
	10.0	46.4 ± 1.7 (12)	4.7	5.5
(C) Normal thymus cells	–	43.0 ± 1.9 (12)	5.9	7.9
	1.0	23.2 ± 2.1 (12)	9.4	31.0

[a] Calculated from percent exchange (this table) and total cell calcium content data of Table I.

in cancer cells, while having this effect on normal cells. This phenomenon may likely be related to a high permeability of cancer cell plasma membrane to external Ca^{2+}. We further investigated this possibility with the experiments shown in Table IV.

Both normal and neoplastic cells have been treated with 1.0 mM iodoacetic acid, a well known inhibitor of the glycolytic pathway, and with 6.7 μM rotenone, which inhibits mitochondrial O_2 uptake, in order to deplete cell energy stores. In all cell types,

TABLE IV

EFFECT OF A23187 UPON TOTAL CALCIUM CONTENT OF EHRLICH ASCITES, THYMOMA ASCITES AND NORMAL THYMUS CELLS TREATED WITH IODOACETIC ACID (1 mM) AND ROTENONE (6.7 μM)

	A23187 (10 μM)	Cell calcium (mmol/kg protein)
(A) Ehrlich ascites tumour cells	–	35.6 ± 2.8 (6) [a]
	+	28.8 ± 2.6 (3)
(B) Ascites thymoma cells	–	45.3 ± 0.8 (8)
	+	45.1 ± 1.4 (8)
(C) Normal thymus cells	–	15.2 ± 0.5 (4)
	+	60.5 ± 1.5 (4)

[a] Mean ± S.E. (number of experiments).

the inhibitors bring about the increase of intracellular total calcium, although to a different extent. Metabolic inhibition and energy depletion determine 10% calcium increase in normal thymocytes and a more conspicuous calcium increase in Ehrlich and thymoma cells (65% and 4-fold, respectively). By contrast, upon addition of A23187, a different pattern develops, namely, the ionophore provokes a 4-fold cell calcium increase in normal thymocytes while it is ineffective both on neoplastic thymocytes and Ehrlich ascites cells.

Cell energy and membrane permeability thus plays a different role in the control of net intracellular calcium content of normal and neoplastic cells. In normal thymocytes energy depletion increases intracellular Ca^{2+} only by 10% while the ionophore exhibits a powerful additional effect, indicating that it is plasma membrane permeability that mainly opposes Ca^{2+} entry into the cell. In neoplastic cells, it is apparent that ionophore treatment does not affect passive Ca^{2+} entry, which has to be considered as already high under physiological conditions.

It could be argued that the lack of effect of A23187 in neoplastic cells indicates that under physiological conditions the cytosol of these cells is in equilibrium with extracellular Ca^{2+}. The experiments shown here do not allow one to draw conclu-

sions on this problem since they do not involve a direct measurement of intracellular Ca^{2+} concentration. However, the calculation of apparent intracellular Ca^{2+} concentration from total calcium (Table I) and intracellular water content (8.9, 5.0 and 5.2 kg H_2O/kg protein in Ehrlich, thymoma and normal thymus cells, respectively) gives a figure of about 2.0 mM in all cell types irrespective of A23187 treatment, except for normal thymocytes, in which the apparent concentration increases to about 7.5 mM upon A23187 addition. The apparent intracellular Ca^{2+} concentration derived from the value of intracellular exchangeable $^{45}Ca^{2+}$ pool is about 1.0 mM in all conditions but in ionophore-treated thymus cells it increases to about 2.0 mM. The exchangeable $^{45}Ca^{2+}$ pool can be considered more similar than total calcium to the free Ca^{2+} pool, but not identifiable with it. However, the apparent concentration calculated in this way, although certainly in excess with respect to the free Ca^{2+} concentration, gives values always lower than the extracellular Ca^{2+} concentration (1.3 mM), except for the normal ionophore-treated thymocytes. These considerations allow one to establish, with a certain degree of confidence, that in the condition of the present experiments, cancer cell intracellular Ca^{2+} concentration is not in equilibrium with extracellular Ca^{2+}. However, in neoplastic cells, the plasma membrane Ca^{2+} permeability is not affected by Ca^{2+} ionophore, so that it can be considered already high under physiological conditions. Ca^{2+} permeability does not represent a limiting step for Ca^{2+} entry into the cell and it is irrelevant for the regulation of steady-state intracellular Ca^{2+} content.

Nevertheless, the uncontrolled inwardly directed leak, supported by the concentration gradient and strengthened by the intracellular negative membrane potential, is likely counteracted by a powerful energy-dependent Ca^{2+} extruding mechanism. Calcium extrusion in Ehrlich ascites cells has been previously shown [7]. Furthermore, the different total cell calcium content between cells under metabolically favourable and energy-depleted conditions (Tables I and IV) indicates that this mechanism could operate also in ascites thymoma cells. On the other hand, the results shown suggest that in normal thymus cells membrane permeability is a more determining feature than energy [14] for the control of steady-state cell calcium content.

Kinetic experiments are in progress to investigate further the nature and meaning of the lack of effect of A23187 on cancer cell calcium content.

The results presented in this paper clearly show that the antibiotic ionophore A23187 is without effect on cancer cells, while it does provoke stimulation of aerobic glycolysis and intracellular calcium increase in normal thymocytes. It must be noted, however, that the control rate of cancer cell aerobic glycolysis is much higher than that of normal cells, although at different levels in the two neoplastic strains.

It is known from other sources that A23187 also induces normal thymus cell mitogenic stimulation and that cell division is permanently activated in neoplastic cells [23,24].

On the basis of these data, we suggest that the antibiotic ionophore A23187 mimics neoplastic transformation in normal thymus cell and that cancer cells are insensitive to the ionophore because they have already acquired features similar to those which A23187 induces in normal cells by permeabilization to Ca^{2+} (viz. permanent mitogenic stimulation and high aerobic glycolysis).

A further aspect to be discussed is that related to the role of monovalent cations in the modulation of the effect of A23187 on cancer cells. Previous results showed, in fact, that A23187 increases cell calcium in Ehrlich ascites tumour cells suspended in medium devoid of Na^+ and K^+ [7,19]. The present results, however do not allow one to draw conclusions on this subject; in particular, it remains unclear whether monovalent cations act by saturation of cation binding sites or by ionic strength and membrane potential modifications.

The last point we would like to discuss is that concerning the role of magnesium in the overall picture. This cation is also directly involved in the regulation of aerobic glycolysis [19,25] as well as in the modulation of cell mitotic activity [26]. As far as glycolysis is concerned, however, it has been shown that Mg^{2+} activity is secondary to that of Ca^{2+} [19]. In the present experiments, the movement of Mg^{2+} seems not to be primarily involved in the stimulation of aerobic glycolysis, as can be seen in Table II. A23187, in fact, decreases thymoma ascites cell magnesium content without affecting lactate accumulation.

Conclusion

In conclusion, the data presented in this paper indicate that the ionophore A23187 increases cell calcium content and stimulates aerobic glycolysis of normal cells. This effect is completely lacking in cancer cells. Furthermore, the results suggest that neoplastic transformation may be ascribed to a cell alteration similar to that induced by Ca^{2+} ionophore in normal cells. The exact nature of this alteration remains unexplained, although the lack of A23187 effect indicates that plasma membrane modifications, leading to derangement of cancer cell calcium content and compartmentalization, are involved.

Acknowledgements

This work is dedicated to Professor T. Terranova, former Director of this Institute, who died on July 10th, 1980; his enthusiastic support was greatly appreciated. Work supported by CNR's Progetto Finalizatto 'Controllo della Crescita Neoplastica'.

References

1 Wenner, C.E. (1975) in Cancer, A. Comprehensive Treatise (Becker, F.F., ed.), Vol. 3, pp. 389–403, Plenum Press, New York
2 Bygrave, F.L. (1976) in Control Mechanisms in Cancer (Criss, W.E., Ono, T. and Sabine, J.R., eds.), pp. 411–453, Raven Press, New York
3 Moore, L. and Pastan, I. (1978) Ann. N.Y. Acad. Sci. 307, 177–193
4 Krebs, E.G. and Fischer, E.H. (1962) Adv. Enzymol. 24, 263–290
5 Gever, W. and Krebs, H.A. (1966) Biochem. J. 98, 720–734
6 Whitfield, J.F., Boynton, A.L., MacManus, J.P., Sikorska, M. and Tsang, B.K. (1979) Mol. Cell. Biochem. 27, 155–179
7 Cittadini, A., Bossi, D., Rosi, G., Wolf, F. and Terranova, T. (1977) Biochim. Biophys. Acta 469, 345–349
8 Cittadini, A., Bossi, D., Wolf, F., Magalini, S., Milani, A. and Terranova, T. (1981) Mol. Cell. Biochem., in the press.
9 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530
10 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977
11 Scarpa, A., Baldossarre, J. and Inesi, G. (1972) J. Gen. Physiol. 60, 735–749
12 Lew, V.L. and Ferreira, H.G. (1978) in Current Topics in Membrane and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 10., pp. 217–277, Academic Press, New York
13 Jensen, P. and Rasmussen, H. (1977) Biochim. Biophys. Acta 468, 146–156
14 Dani, A.M., Cittadini, A., Calviello, G., Festuccia, G. and Terranova, T. (1978) Mol. Cell. Biochem. 22, 139–145
15 Cittadini, A. and Van Rossum, G.D.V. (1978) J. Physiol. 281, 29–43
16 Ferreira, H.G. and Lew, V.L. (1975) Nature 259, 47–49
17 Flatman, P.W. (1980) J. Physiol. 300, 19–30
18 Cittadini, A., Bossi, D., Longhi, G. and Terranova, T. (1975) Mol. Cell. Biochem. 8, 49–57
19 Bossi, D., Cittadini, A., Wolf, F., Milani, A., Magalini, S. and Terranova, T. (1979) FEBS Lett. 104, 6–12
20 Hume, D.A., Radik, J.L., Ferber, E. and Weidemann, M.J. (1978) Biochem. J. 174, 703–709
21 Warburg, O. (1931) The Metabolism of Tumours (Smith, R.R., ed.), Academic Press, New York
22 Bygrave, F.L. (1967) Nature 214, 667–671
23 Luckasen, J.R., White, J.G. and Kersey, J.H. (1974) Proc. Natl. Acad. Sci. USA 71, 5088–5090
24 Ambrose, E.J. (1975) in Biology of Cancer (Ambrose, E.J. and Poe, F.J.C., eds.), 2nd edn. Halsted Press, London
25 Krebs, H.A. (1972) Essays Biochem. 8, 1–34
26 Rubin, H. (1975) Proc. Natl. Acad. Sci. USA 72, 3551–3555