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CALCIUM AND A_{23187}-INDUCED CYTOLYSIS OF MOUSE THYMOCYTES

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SUMMARY : The cytotoxic effects of ionophore A_{23187} were studied in parallel with its action on calcium uptake in isolated mouse thymocytes. Under conditions where the cells were preincubated in a calcium-containing medium prior to ionophore treatment a close relationship could be observed between the extent of cell lysis and the stimulation of calcium uptake in the presence of A_{23187}. In addition, increasing concentrations of calcium ions in the incubation medium lead to a pronounced decrease of cell viability and to a stimulation of calcium uptake suggesting that calcium is critical for cell survival.

The use of ionophores, i.e. of molecules able to alter ionic permeability of artificial and biological membranes has allowed the study of the role of ions in cell proliferation, particularly the role of calcium in lymphoblast-transformation. A_{23187}, a carboxylic antibiotic which transports essentially calcium, has been shown to stimulate the proliferation of peripheral human lymphocytes and pig lymph node lymphocytes. This effect is blocked in the presence of either EGTA or lanthanum ion (1-6). An increase in intracellular calcium has therefore been postulated to represent one of the first events triggering mitogen-induced DNA synthesis (1, 2, 4, 6). Hesketh et al (7), however have demonstrated that the elevation of the intracellular calcium concentration occurs at mitogen doses markedly higher than those necessary to induce blast-transformation. At concentrations higher than 10^{-6}M, the ionophore A_{23187} appears to be actually cytotoxic, with some differences in sensitivity among the species (1, 2, 4, 5, 7). Recently, Kaiser & Edelman, (8, 9) have suggested that the lytic effects of glucocorticoids on rat thymic lymphocytes could be similar to those of the ionophore and thus mediated by an increase of cell membrane permeability to calcium. This ion therefore appears to play a key role in cell metabolism and differentiation, and possibly in steroid hormone action. In order to further define the cytotoxic action of calcium we have studied, in lymphoid cell suspensions the effect of calcium A_{23187} on cell viability and also the ^{45}calcium uptake in the presence of extracellular calcium and/or ionophore.

MATERIALS AND METHODS

Animals and reagents

Adrenalectomy has been shown to decrease the proportion of thymocytes which spontaneously die during in vitro incubation (10, 11). The animals

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used in our investigations (female C_{57} BL/6 mice, 6-8 weeks old) were thus routinely adrenalectomized under pentobarbitone anesthesia 4-5 days before each experiment and given 1 % saline as drinking water. Ionophore A_{23187} was a gift from Elli Lilly and Co (Indianapolis, Indiana, U.S.A.). The drug was dissolved in absolute ethanol and diluted to the appropriate concentrations before each experiment. The final ethanol concentration was always lower than 1 % and did not alter cell viability.

Culture medium (minimum essential medium, MEM, calcium free, ref. 138) and the various supplements (sodium pyruvate, L-glutamine, penicillin-streptomycin and non-essential amino acid solution) were obtained from Gibco. Sterile ^{45}calcium chloride solution (20-25 µCi/g) was purchased from C.E.A. (Saclay, France).

Isolation of thymocytes

The procedure for thymocyte isolation has been previously described in detail (12, 13). Cell suspensions were usually adjusted to contain 10-30 X 10^6 cells/ml in MEM supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), 1 % (v/v) non essential amino-acid solution, 100 U/ml penicillin and 100 µg/ml streptomycin (medium A). Cell preparation was carried at room temperature and the cell suspensions were preincubated at 37°C for 30 min. before the start of the experiments.

Determination of cell viability

Viability of the cells was estimated by the trypan blue exclusion technique and was always over 95 % at the end of the isolation procedure (14).

Measurement of Calcium uptake

0.1 ml of cell suspension (30 X 10^6 cells/ml) was added to glass tubes containing medium A supplemented with various concentrations of Ca^{++}, 0.5µCi of ^{45}Ca and various concentrations of ionophore A23187 in a final volume of 0.5 ml.

After incubation at 37°C or 4°C for various periods of time, the tubes were put into ice and rapidly filtered on Whatman GF/A filters. The filters were washed with 3 X 10 ml of ice cold buffer (135 mM NaCl, 5 mM KH_2PO_4, 5 mM Tris-HCl, 1 mM $MgCl_2$, 5 mM glucose pH 7,4) and the radioactivity collected on the filters counted by liquid scintillation spectrometry.

RESULTS

1. ^{45}calcium uptake and cytotoxic effects in the presence of ionophore A23187

Preliminary experiments have shown that this ionophore does not exert any toxic effect in the absence of calcium (i.e. in a medium containing 1 mM EGTA) even at a concentration of 10^{-5}M, which in the presence of calcium induces almost complete cell death after 60 min incubation. We have also confirmed that both ionophore-induced cytotoxic effect and ^{45}calcium uptake are highly dependent on the temperature, being almost completely inhibited in experiments performed at 4°C (9, 15, 16).

The kinetics of the effects of various concentrations of ionophore A_{23187}, after preincubation of the cells at 37°C for 30 min. in a medium supplemented with 1 mM Ca^{++}, are shown in figure 1. The stimulation of ^{45}Ca uptake was dependent upon the concentration of ionophore used : at 10^{-7}M no stimulation could be observed, whereas the percentage stimulation reached about

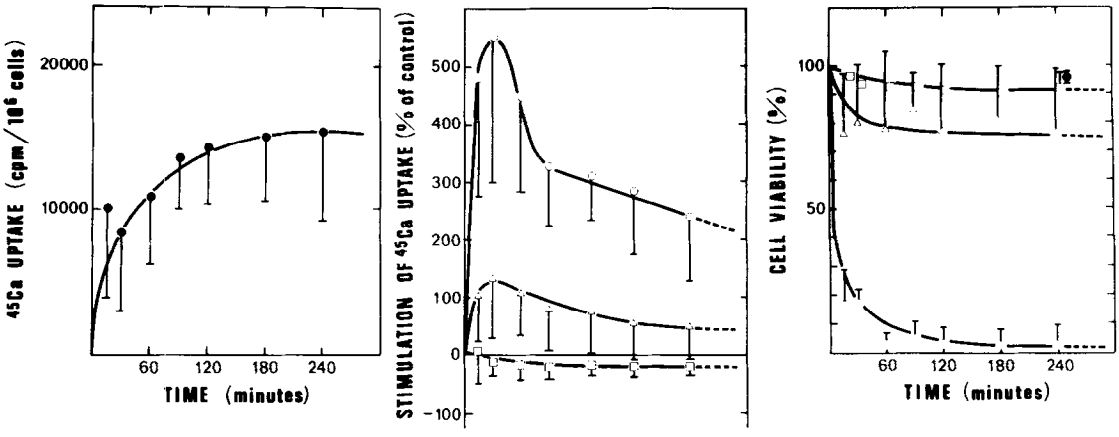

Figure 1 : Dose dependency of ionophore effects.

Left panel : Kinetic of ^{45}Ca uptake in the absence of ionophore (control).

Middle panel : Ionophore induced stimulation of ^{45}Ca uptake (values of uptake in the absence of ionophore are taken as baseline). (□) 10^{-7}M A_{23187}, (△) 10^{-6}M A_{23187}, (○) 10^{-5}M A_{23187}.

Right panel : Cytotoxic effects of various concentrations of ionophore A_{23187} (□) 10^{-7}M, (△) 10^{-6}M and (○) 10^{-5}M ionophore A_{23187}. Each value is the mean (± SD) of triplicate determinations in 4 different experiments.

500 % in the presence of 10^{-5}M A_{23187}. These pattern of stimulation were transient with a maximum at 30-45 min and a gradual decline to control values, and resemble those described by Jensen & Rasmussen (17) in human peripheral lymphocytes. Under these experimental conditions, the cytolytic effects of ionophore were also concentration dependent (figure 1, right panel).

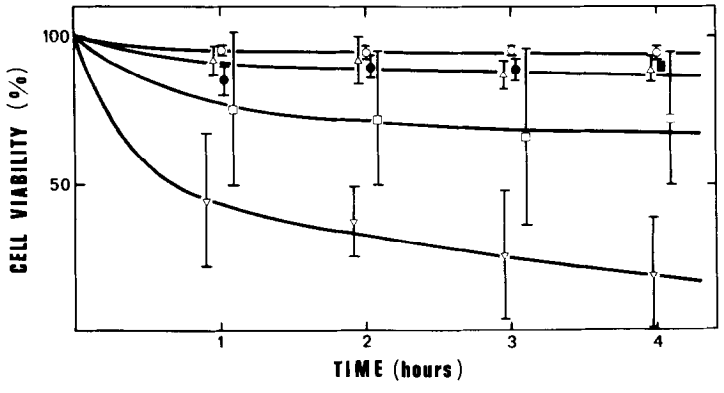

Figure 2 : Kinetics of calcium-induced cell lysis (○) calcium free medium, (●) EGTA, 1 mM, (△) calcium, 1 mM, (□) calcium, 2 mM, (▽) calcium, 3 mM. Each value is the mean (± SD) of 4 experiments.

2. Calcium-dependent cytolysis and ^{45}calcium uptake in thymocytes

Calcium appears to play a key role in the ionophore-mediated lytic process. This cation was also demonstrated to exert a lytic effect at high concentration (9). We thus studied the effect of various extracellular concentrations of calcium on cell viability and ^{45}calcium uptake in mouse thymocytes.

As shown in figure 2 increasing concentrations of calcium chloride induced a pronounced and rapid decrease in cell viability.

Calcium toxicity was not related simply to an increase in the ionic strenght of the medium but appeared specific to the cation. Indeed mouse thymocytes remained viable even in the presence of 5 mM Mg^{++} (results not shown).

The rise of the extracellular calcium concentration appear also associated with a marked increase of cellular ^{45}calcium uptake (table I).

DISCUSSION

The ionophore A_{23187} exhibits in vitro cytotoxic effects on mouse thymocytes. The properties of the drug action reported here are comparable with those previously described in rat thymocytes (8, 9, 18) and human or pig peripheral lymphocytes (4, 5, 7). Mouse thymocytes appear particularly sensible to ionophore action. At concentrations which were shown to be mitogenic in other species and cell types, is still exerts in our experiments, a perceptible effect on cell viability.

Several arguments suggest that the toxicity of ionophore could be related to the extent of calcium uptake :

- at low temperature (4°C) or in the presence of EGTA (results not shown) both the calcium uptake and the ionophore activity were markedly decreased.
- Several authors have previously demonstrated an ionophore-induced stimulation of calcium uptake (4, 7, 8, 17, 19, 20) as well as toxic effect of A_{23187} (1, 2, 4, 5, 8, 9, 18). We showed, in addition, that these two effects of the drug exhibit the same dose-effect curve.

The time course of the stimulation of calcium uptake in the presence of ionophore was characterized by a transient peak, followed by a gradual return to control values. This pattern is similar to that described by Jensen & Rasmussen (17) in human peripheral lymphocytes.

This biphasic ionophore stimulation of calcium uptake could be attributed to a rapid effect increasing the permeability of the cell membrane and then to an action on the rate of efflux of calcium from mitochondria thus preventing net cation accumulation by the cells (17, 21, 22, 23). This hypothesis is supported by observations showing mitochondrial alterations in lymphocytes treated by A_{23187} (2).

Table I : Calcium uptake as a function of the extracellular calcium concentration.

Extracellular Calcium	Time (minute)					
	10	20	30	40	60	120
1 mM	3004 ± 1735	3635 ± 2270	4178 ± 2575	7143 ± 6194	7727 ± 6521	17390 ± 13306
2 mM	42458 ± 10949	44765 ± 11231	48933 ± 9857	54519 ± 9350	55223 ± 9186	51900 ± 8679
3 mM	67558 ± 12390	67940 ± 16964	72081 ± 15793	80105 ± 21416	76123 ± 23561	76116 ± 20716

The role of calcium ion appears rather complex. This cation is considered to be important in controlling the proliferation capacity of lymphocytes (24). We also demonstrated that the presence of 1 mM Ca^{++} improved the long-term survival of mouse thymocytes and the levels of spontaneous thymidine incorporation (results not shown). A similar result was described for uridine incorporation by Kaiser & Edelman (8). Calcium has been shown to induce a membrane hyperpolarisation (25, 26) important for triggering calcium-induced mitosis (26).

However, in the presence of higher calcium concentrations (2-3 mM) almost 50 % of the mouse thymocytes are rapidly lysed. This effect is specific in the sense that it does not occur in the presence of up to 5 mM magnesium. The time course pattern of calcium-induced cell death is biphasic suggesting that all the thymocytes are not equally susceptible to calcium action. This heterogeneity could reflect the well known heterogeneity of thymus populations (12, 27, 28).

The existence of a calcium threshold above which this cation becomes inhibitory rather than stimulatory could be therefore postulated. Under normal conditions the intracellular concentration of ionized calcium is assumed to be well below this cytotoxic level (18, 29, 30), whereas the presence of ionophore A_{23187} or a high extracellular calcium concentration would lead to an increase in cytoplasmic free calcium above this level. The mechanism of cell death induced in the presence of high concentrations of calcium could be attributed to several processes such as inhibition of Na-K ATPase (31, 32), Ca ATPase (33) and also diminution of ATP production (33). All these parameters have indeed been demonstrated to be inhibited by increasing amounts of calcium and to lead to a change in intracellular ionic and water content (9).

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