Proc. Nat. Acad. Sci. USA
Vol. 71, No. 12, pp. 5088–5090, December 1974

Mitogenic Properties of a Calcium Ionophore, A23187

(calcium fluxes/mitogenic signals/human lymphocytes)

JOHN R. LUCKASEN, JAMES G. WHITE, AND JOHN H. KERSEY

Departments of Laboratory Medicine and Pathology, Dermatology, and Pediatrics, University of Minnesota, Minneapolis, Minn. 55455

Communicated by C. J. Watson, October 15, 1974

ABSTRACT A23187 is a carboxylic antibiotic that selectively transfers calcium, magnesium, and other divalent cations across biologic membranes. This ionophore was found to produce morphologic blast transformation, DNA synthesis, and mitosis in human lymphocytes. Several hours of ionophore–cell contact were necessary to produce optimal mitogenesis. The effects were highly dependent on the presence of extracellular calcium and much less dependent on extracellular magnesium. Lanthanum chloride prevented the development of the observed ionophore effects.

Results are consistent with the hypothesis that under physiologic conditions the interaction of antigens or mitogens with specific receptors at the lymphocyte membrane initiates events that alter calcium fluxes and result in increased cytoplasmic calcium. Increased cytoplasmic calcium is postulated to play a central role in the generation of surface-to-nuclear signals that initiate the process of DNA synthesis and cell division.

Lymphocytes may be induced to undergo mitogenesis by a number of agents, including plant lectins. Lectins, such as phytohemagglutinin (PHA), exert their effect at the cell membrane (1, 2) and result in a number of biochemical changes within minutes after ligand binding. Some of these changes are enhanced membrane lipid fluidity (3, 4), increased cellular cyclic GMP (5), and enhanced uptake of amino acids and ions, including calcium (6, 7). The role of each of these alterations in generation of mitogenic signals that are transmitted from the cell surface to the nucleus is not clear. Some evidence suggests that calcium may be of central importance; extracellular calcium has been shown to be necessary for proliferation in lymphocytes stimulated by PHA (8, 9) and phorbol myristate acetate (10).

Ionophorous antibiotics modify the transport of specific ions across biological membranes. A23187 is a carboxylic acid antibiotic derived from cultures of *Streptomyces chartreusensis* that transports divalent cations across biologic membranes (11). A23187 has highest affinity for calcium, and somewhat less for magnesium and other divalent cations (11). A23187 has recently been demonstrated to activate several calcium-dependent biologic processes, including DNA synthesis in sea urchin eggs (12), secretion and aggregation in platelets (13, 14), and secretion in mast cells (15, 16). We postulated that, similarly, A23187 would stimulate lymphocytes to mitogenesis in a calcium-dependent manner. Our results indicate that A23187 can induce blast transformation, DNA synthesis, and mitosis, and that these effects are not found when calcium is absent from the external media.

Abbreviation: PHA, phytohemagglutinin

Address reprint requests to: John H. Kersey, 184M Jackson Hall, University of Minnesota, Minneapolis, Minn. 55455.

MATERIALS AND METHODS

These studies utilized human peripheral blood lymphocytes separated by standard Ficoll–Hypaque density gradient centrifugation. Separated cells were washed and resuspended in RPMI-1640 medium with 10% heat-inactivated (56° for 30 min) fetal calf serum, supplemented with penicillin and streptomycin, with or without calcium salts (Associated Biomedic Systems, Buffalo, N.Y.). A23187 (courtesy of Eli Lilly and Co., Indianapolis, Ind.) was prepared in a stock solution of 10 mM in dimethylsulfoxide, stored at −70°, and appropriate concentrations were prepared with medium. One hundred thousand separated and washed cells, mixed with A23187 or an optimal concentration of PHA-P (7.5 μg/ml; Difco, Detroit, Mich.) in 200-μg volumes, were placed into wells of microplates. Plates were incubated at 37° in a humidified atmosphere with 5% CO_2. For studies of DNA synthesis, wells were pulse-labeled with 5 μCi of [^{3}H]thymidine (specific activity 13 Ci/mmol) on the 67th hr of incubation. At 72 hr, contents were transferred to glass filter paper with a multiple cell culture harvester and counted in a liquid scintillation counter. All determinations were based on five replicate wells. Uptake of [^{3}H]thymidine is expressed as counts per minute (cpm) ± standard error of the mean (SEM). In cell washing experiments, separated lymphocytes were incubated with ionophore for various lengths of time and washed three times with excess medium.

The percentage of lymphoblast transformation (i.e., large morphologically distinct cells with reticular chromatin and basophilic cytoplasm) and number of cells in metaphase was determined from Wright–Geimsa stained preparations.

RESULTS

The effect of various concentrations of A23187 on lymphocytes in 72-hr culture was studied by blast transformation, incorporation of [^{3}H]thymidine into DNA, and formation of mitoses. The results shown in Fig. 1 demonstrate that the peak effect was at 10^{-6} M, with 54% blast transformation and 59,099 ± 1470 cpm uptake of [^{3}H]thymidine. A smaller but significant response was also noted at 5×10^{-7} M. Additional experiments (not shown) indicate an increase in the number of cells in metaphase from 1 (control) to 80 (ionophore-treated) cells in metaphase per 50 high-power microscope fields at 72 hr of culture at the optimal concentration of A23187. At concentrations higher than 10^{-6} M, the ionophore usually resulted in significant cell death. Dimethylsulfoxide alone had no effect in these experiments at concentrations equal to those effective in the ionophore–dimethylsulfoxide experiments.

Studies were performed to determine the time of ionophore–lymphocyte contact necessary for optimal stimulation. In these experiments, cells were left in contact with A23187 for

various periods and then washed as described in *Materials and Methods*. Table 1 indicates that a small response was evident within 2.5 hr of contact (1260 ± 180 cpm). The response was increased significantly at 24 hr of contact (26,500 ± 2180 cpm), and maximal at 72 hr (39,000 ± 1680 cpm).

Additional studies were then designed to determine the influence of extracellular calcium and magnesium on the effects of A23187. The experiments, as shown in Table 2, indicate that the effects of the ionophore were markedly reduced in calcium-free RPMI-1640 medium (34% of control). EDTA was added in one group to bind divalent cations (including calcium) present in the fetal calf serum which is required for cell viability in 72-hr cultures of human lymphocytes; this further decreased the response to 7% of control. As shown, addition of calcium nitrate to calcium-free RPMI reestablished the ionophore effect. Results with standard RPMI-1640 medium (containing 4×10^{-4} M Ca^{++}) were approximately equal to those with equivalent additions of Ca^{++}. A higher calcium concentration (10^{-3} M Ca^{++}) resulted in the greatest uptake of [³H]thymidine (161% of the RPMI-1640 response). As shown, uptake of [³H]thymidine was inhibited to a much lesser degree in magnesium-free medium (75% of the RPMI-1640 response) compared to calcium-free medium (34% of the RPMI-1640 response). Lanthanum inhibits calcium transport, competes with calcium for ionophore binding sites, and inhibits ionophore stimulation of respiration in mitochondria (11). We found that lanthanum (as chloride), when present in the medium with equimolar concentrations of calcium (as nitrate), completely abrogated the ionophore stimulation of [³H]thymidine uptake.

In these experiments the degree of lymphocyte stimulation was always significantly less than that seen with PHA-P (Difco) at 72 hr. The percentage of blast transformation was about 50% of that seen with PHA-P (50% compared to 90%). Uptake of [³H]thymidine was generally about 25% of that with PHA-P (about 40,000–60,000 cpm compared to about 200,000 cpm).

DISCUSSION

Results of these experiments indicate that A23187, an ionophore with a high affinity for calcium (11), is mitogenic for human lymphocytes. These results were of interest in light of observations indicating that the same ionophore stimulated calcium-dependent secretory processes in mast cells (15, 16) and platelets (13, 14) and DNA synthesis in sea urchin eggs (12). Our results indicated that calcium deprivation largely abrogated the ionophore effect, while magnesium deprivation only slightly abrogated the effect. Previously, Alford showed that calcium deprivation had a much greater effect than magnesium deprivation in nullifying mitogenesis induced by PHA (8).

Increased uptake of exogenous calcium has been observed in lymphocytes stimulated with the plant lectin, PHA (6, 7) and our preliminary observations (unpublished) indicate that A23187 similarly enhances cellular uptake of extracellular calcium.

Our data are consistent with the hypothesis that interaction of antigens and mitogens with specific receptors at the cell surface initiates a series of events resulting in net influx of calcium (in the direction of a large calcium gradient), resulting in an increase in concentration of calcium in the cytoplasm. The increase in cytoplasmic calcium is postulated to be at least part of the mitogenic signal(s) which then results in DNA synthesis and cell division.

In these experiments, optimal mitogenic effects were observed only after several hours of contact between lymphocytes and the ionophore. The reasons for these observations are not clear, but could be interpreted to indicate that a period of time is required for the ionophore to penetrate into the cell and that at least some of the effects are due to release of calcium from intracellular stores. Ionophore-induced DNA synthesis in sea urchin eggs and platelet aggregation and secre-

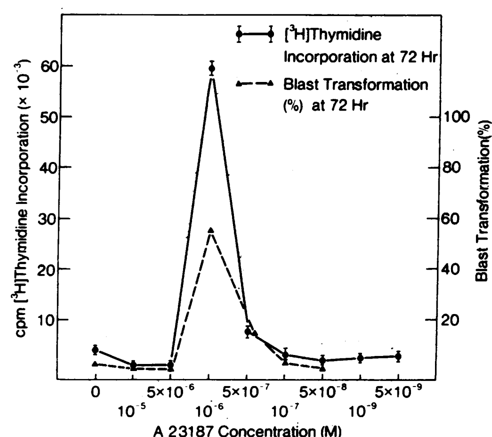

FIG. 1. Dose-response for ionophore-induced blast transformation and uptake of [³H]thymidine in RPMI-1640 medium.

TABLE 1. *Lymphocyte response with various times of incubation with A23187*

Time of contact of ionophore (10^{-6} M) with cells	Incorporation of [³H]thymidine at 72 hr (net cpm ± SEM)	% Maximal (72-hr) response
10 min	418 ± 272	1
30 min	0	0
1 hr, 30 min	0	0
2 hr, 30 min	1,260 ± 183	3
24 hr	26,500 ± 2,180	67
72 hr	39,200 ± 1,680	100

TABLE 2. *Influence of calcium and magnesium on effects of A23187*

Culture medium for lymphocytes	Uptake of [³H]thymidine (net cpm ± SEM)	% Response compared to control (RPMI-1640)
RPMI-1640*	40,083 ± 986	—
Calcium-free RPMI-1640	13,489 ± 1,055	34
+ EDTA (3×10^{-4} M)	2,886 ± 321	7
+ Ca^{++} (3×10^{-5} M)	29,151 ± 1,150	73
+ Ca^{++} (1×10^{-4} M)	42,731 ± 2,370	107
+ Ca^{++} (3×10^{-4} M)	47,723 ± 3,727	119
+ Ca^{++} (1×10^{-3} M)	64,396 ± 3,364	161
+ Ca^{++} (3×10^{-3} M)	36,420 ± 1,589	91
Magnesium-free RPMI-1640	30,142 ± 1,144	75

* 10% heat-inactivated calcium-containing fetal calf serum in all groups. Calcium was added as calcium nitrate.

tion do not require extracellular calcium, and in these systems A23187 was postulated to induce calcium release from intracellular stores (12–14). Alternatively, it is possible that internal calcium stores are not involved in lymphocyte mitogenesis, but that intracellular calcium alterations due to influx from the extracellular medium for prolonged periods result in optimal mitogenic effects.

We thank R. Estensen and H. Kunkel for advice and encouragement, A. Sabad for technical assistance, and S. Perry for assistance with the manuscript. The work was supported by grants and a contract from the U.S. Public Health Service.

1. Anderson, J., Edelman, G. M., Möller, G. & Sjöberg, O. (1974) *Eur. J. Immunol.* **2**, 233–236.
2. Greaves, M. F. & Bauminger, S. (1972) *Nature New Biol.* **235**, 67–69.
3. Ferber, E., Reilly, C. E., de Pasquale, G. & Resch, K. (1974) in *Proc. Eighth Leucocyte Culture Conf.* (Academic Press, New York), pp. 529–534.
4. Barnett, R. E., Scott, R. E., Furcht, L. T. & Kersey, J. H. (1974) *Nature* **249**, 465–466.
5. Hadden, J. W., Hadden, E. M., Haddox, M. K. & Goldberg, N. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3024–3027.
6. Allwood, G., Asherson, G. I., Davey, M. J. & Goodford, P. J. (1971) *Immunology* **21**, 509–516.
7. Whitney, R. B. & Sutherland, R. M. (1972) *Cell. Immunol.* **5**, 137–147.
8. Alford, R. H. (1970) *J. Immunol.* **104**, 698–703.
9. Whitney, R. B. & Sutherland, R. M. (1972) *J. Cell. Physiol.* **80**, 329–337.
10. Whitfield, J. F., MacManus, J. P. & Gillan, D. J. (1973) *J. Cell. Physiol.* **82**, 151–156.
11. Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977.
12. Steinhardt, R. A. & Epel, D. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1915–1919.
13. Feinman, R. D. & Detwiler, T. C. (1974) *Nature* **249**, 172–173.
14. White, J. G., Rao, R. H. R. & Gerrard, J. M. (1974) *Amer. J. Pathol.*, in press.
15. Foreman, J. C., Mongar, J. L. & Gomperts, B. D. (1973) *Nature* **245**, 249–251.
16. Cochrane, D. E. & Douglas, W. W. (1974) *Proc. Nat. Acad. Sci. USA* **74**, 408–412.