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A POSTULATED MECHANISM FOR THE COORDINATE EFFECTS OF IONOPHORE A23187 ON CALCIUM UPTAKE AND CELL VIABILITY IN RAT THYMOCYTES

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At the physiological concentration of Ca^{2+}, the presumed calcium ionophore A23187 produced dose-related increases in ^{45}Ca uptake by rat thymocytes and decreases in cell viability, effects that displayed a strong linear correlation. In media containing a very low concentration of Ca^{2+} ($7 \cdot 10^{-6}$ M), in contrast, ionophore A23187 had no specific effect on either ^{45}Ca uptake or cell viability. The calcium-dependent cytotoxicity of ionophore A23187 resembles that of other agents that are not ionophores, but that are known to perturb the plasma membrane. Consequently, we suggest that, in the rat thymocyte, ionophore A23187 may not act as a true ionophore, but may perturb the cell membrane, allowing Ca^{2+} to pass freely through the membrane along its electrochemical gradient.

Introduction

It has been suggested that calcium plays a major role in the activation of many biological systems, among them muscle contraction, exocrine and endocrine secretion, cyclic nucleotide metabolism, and the cellular transport of various substrates [1]. The antibiotic A23187 has a high affinity for calcium and other divalent cations, for which it is thought to act as an ionophore [2,3]. It has been considered, therefore, a potentially useful probe for studying the role of calcium in the regulation of various biological systems. A monocarboxylic acid (M_r 523) obtained from cultures of *Streptomyces chartreusensis*, A23187, has been shown to enhance the passage of divalent cations along their electrochemical gradients across biological membranes. In the presence of extracellular calcium, ionophore A23187 mimics the activity of various agents that alter certain physiological functions, and this has been taken as evidence that changes in intracellular calcium are involved in the activity of these agents [1].

One of the effects of ionophore A23187 is to increase the uptake of the glucose analogue, 3-*O*-methylglucose, by rat thymocytes in vitro, an effect which mimics that of the mitogen concanavalin A [4,5]. We have recently demonstrated that the thyroid hormone, 3,5,3′-triiodothyronine (T_3), increases the uptake of calcium and of the sugar analogues, 2-deoxy-D-glucose and 3-*O*-methylglucose, in isolated rat thymocytes [6,7]. We have also shown that the effect of T_3 on sugar uptake requires extracellular calcium [7]. Consequently, we undertook studies to determine whether A23187 can mimic the effect of T_3 in this system.

Methods

Measurement of ^{45}Ca uptake. Thymocytes were isolated form weanling female CD rats (Charles River Breeding Laboratories) according to a method that we have described previously [6]. Isolated cells were suspended in 3 ml of Krebs-Ringer-25 mM Tris buffer, pH 7.4, in a final

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concentration of $45 \cdot 10^6$ cells/ml. In most experiments, three types of medium were employed: (a) standard medium (Krebs-Ringer-25 mM Tris containing 1 mM Ca^{2+}); (b) Ca^{2+}-free medium (the same as standard medium but without added calcium salts; this contained $5 \cdot 10^{-6}$ M Ca^{2+}, representing contaminants of the other salts added); and (c) EGTA-medium (Ca^{2+}-free medium containing 1 mM EGTA). Ionophore A23187 (kindly donated by Dr. R.L. Hamill, Eli Lilly, Co., Indianapolis, IN) in a concentration of 0.1 M was freshly dissolved in undiluted (100%) dimethylsulfoxide (DMSO). Cells were allowed to equilibrate at 37°C in air for 30 min. A23187 in DMSO, as well as DMSO alone, was then added in various concentrations prior to or concomitant with ^{45}Ca (2 μCi/ml, S.A. 884 Ci/mol, New England Nuclear Corp., Boston, MA). At the end of the incubation period, aliquots of 200 μl were quickly removed into microtubes and centrifuged at 10000 rev./min (Beckman microfuge) for 30 s. The supernatant was aspirated. The cell pellet was transferred into vials containing toluene-Triton X-100, and its ^{45}Ca content measured in a liquid scintillation counter with automatic quench correction. By means of [^{3}H]mannitol, values were corrected for the extracellular fluid occluded in the cell pellet, as we have previously described [6].

Cell viability. To assess cell viability, trypan blue (final concentration, 0.3 gm/dl) was added to cells at the end of incubations, and 2–5 min later the percentage of cells that remained viable, as judged from their ability to exclude the dye, was determined by light microscopy.

Statistical analysis. Where appropriate, statistical differences between experimental groups were evaluated using Dunnet's test for comparisons between multiple samples and a single control. Analysis of variance followed by Newman-Keuls multiple range test was employed for comparisons among multiple groups [8].

Results and Discussion

Effects on Ca uptake

In initial experiments, thymocytes were suspended in standard, Ca^{2+}-free, or EGTA-medium. DMSO or A23187 were added together with ^{45}Ca (2 μCi/ml), and 1 min and 35 min later cellular content of ^{45}Ca was measured. The basal uptake of ^{45}Ca in cells suspended in standard and Ca^{2+}-free medium was at 1 min of incubation 0.247 ± 0.020 (nmol/10^9 cells, mean ± S.D.) and 0.677 ± 0.035, respectively, and at 35 min 0.251 ± 0.014 and 0.703 ± 0.028, respectively. When A23187 was added in the absence of DMSO, a cloudy suspension was obtained and no effect on ^{45}Ca uptake or cell viability was observed. Therefore, in the remaining experiments, A23187 was dissolved in DMSO prior to its addition ot the media so that, in most experiments, the final concentration of DMSO was 0.01%. In concentrations of 1.0 and 10.0%, DMSO alone produced time- and dose-dependent, calcium-independent increases in ^{45}Ca uptake and decreases in cell viability (data not shown). However, the 0.01% concentration of DMSO routinely employed to dissolve A23187

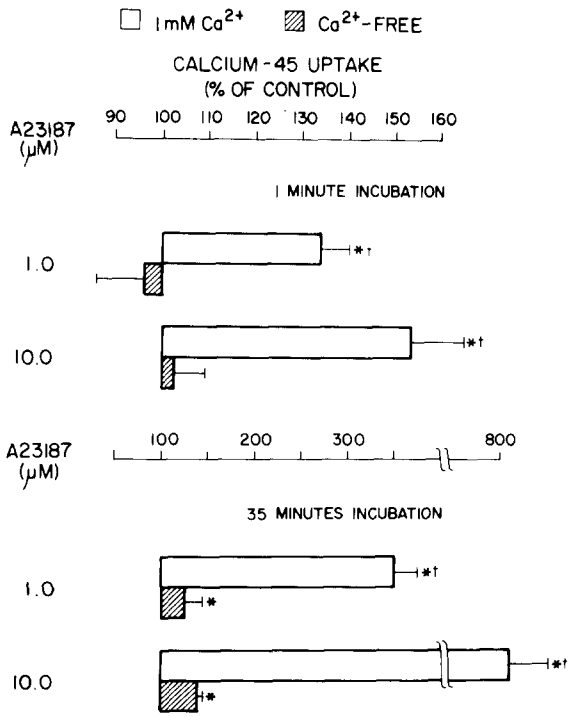

Fig. 1. The effect of ionophore A23187 on calcium-45 uptake in rat thymocytes in vitro. Cells suspended in standard or Ca^{2+}-free medium were incubated with A23187 and ^{45}Ca for 1 min (upper panel) or 35 min (lower panel),and their content of ^{45}Ca was then assessed. Values shown are mean ± S.D. of those obtained in four separate experiments in which triplicate samples in each experimental group were studied. * $P<0.05$ vs. control, † $P<0.05$ vs. corresponding group incubated in Ca^{2+}-free medium.

had neither of these effects when added alone.

A23187 dissolved in DMSO produced a dose- and time-dependent increase in cellular ^{45}Ca uptake in the standard medium (Fig. 1). In Ca^{2+}-free medium, A23187 produced a much smaller, though statistically significant ($P < 0.05$), increase in ^{45}Ca uptake. The effect seen in Ca^{2+}-free medium was probably not specific, since it was small in magnitude and was not dose-related.

The lack of a specific effect of A23187 on ^{45}Ca uptake in the Ca^{2+}-free medium was somewhat surprising. A23187 has been thought to be a true ionophore for Ca^{2+}, and thus to serve as a carrier that facilitates the transport of calcium through the membrane [2,9]. It was expected, therefore, that A23187 in Ca^{2+}-free medium would induce an increase in cellular ^{45}Ca uptake similar to, and probably greater than, that seen in the standard medium.

Effects on cell viability

It has recently been shown that A23187 dissolved in ethyl alcohol produces a marked decrease in the viability of rat thymocytes [10,11]. Therefore, we undertook studies to ascertain the manner in which the effect of A23187 on cellular calcium metabolism and its effect on cell viability might be related. Thymocytes were suspended in the three media and the effects of A23287 on cell viability were assessed. A23187 induced a time- and dose-dependent decrease in cell viability (Figs. 2 and 3). Like its effect on ^{45}Ca uptake, the effect of A23187 on cell viability was calcium-dependent. The small, though significant ($P < 0.05$), effect of A23187 on cell viability in Ca^{2+}-free medium, like its effect on Ca^{2+} uptake, was probably not specific, since it too was small and was not dose-related. On the other hand, it may have been made possible by the small amount of contaminating calcium ($5 \cdot 10^{-6}$ M), since a significant effect of A23187 was not observed in the calcium-free medium that also contained EGTA. Our findings are consonant with those seen in other cell systems, in which the cytotoxic effect of A23187 proved to be calcium-dependent [10–15].

Comparison of the effects of A23187 on ^{45}Ca uptake and cell viability revealed an inverse linear relationship between the increase in ^{45}Ca uptake and the decrease in cell viability (Fig. 4).

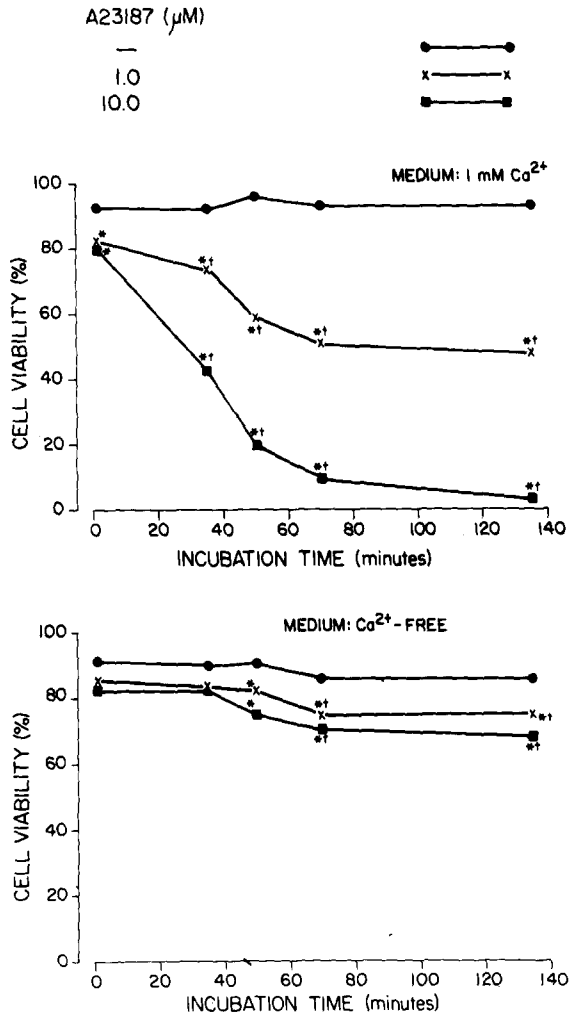

Fig. 2. Time- and dose-dependence of the effect of ionophore A23187 on the viability of rat thymocytes. Cells were suspended in standard medium or Ca^{2+}-free medium and were incubated with or without A23187 for various periods. Their viability was then assessed. Values shown are the mean of those obtained in four separate experiments in which duplicate samples for each experimental group were studied. * $P<0.05$ vs. values in A23187-free controls; † $P<0.05$ vs. values in same groups at zero-time.

Mechanism of action of A23187

Schanne and co-workers [12], in studies of rat hepatocytes, demonstrated that not only A23187 but also nine other compounds structurally unrelated to one another were capable of decreasing cell viability, an effect that was calcium-dependent. The only known common characteristic of these

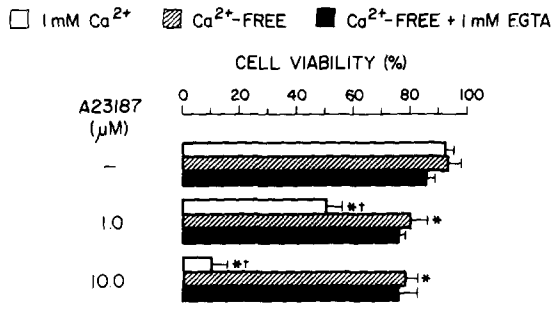

Fig. 3. Calcium-dependence of the effect of ionophore A23187 on the viability of rat thymocytes. Cells suspended in the three different media were incubated with or without A23187, and 70 min later their viability was examined. Values shown are mean ±S.D. of those obtained from three separate experiments in which duplicate samples in each experimental group were studied. * $P<0.05$ vs. values in control samples lacking A23187 and incubated in the same medium; † $P<0.01$ vs. values in cells incubated in the same concentration of A23187, but in the Ca^{2+}-free media with or without EGTA.

nine agents is that they all interact strongly with plasma membranes and induce configurational changes therein. Schanne and co-workers suggested, therefore, that A23187 like the other agents, disrupts the plasma membrane, permitting an increase in calcium uptake and cellular Ca^{2+} concentration that is followed by cell death. Consistent with this view is the ability of A23187 to alter the morphology of the plasma membrane in cultured pig lymphocytes [15], human blood

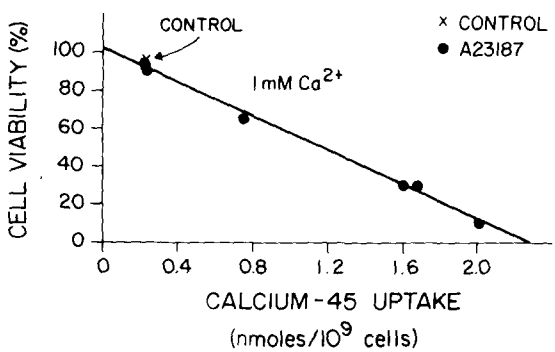

Fig. 4. The effect of ionophore A23187 in rat thymocytes: inverse relationship between cell viability and calcium-45 uptake. Cells suspended in standard medium were preincubated for 30 min with and without A23187. ^{45}Ca was then added and 10 min later ^{45}Ca uptake and cell viability were determined. Values for each point shown are the mean of those obtained in four separate experiments. $r=0.998$, $P<0.01$.

platelets [16], and human erythrocytes [17]. Our own data are entirely in accord with this view. In addition, however, they appear to shed new light upon the manner in which these effects of A23187 are brought about, at least in the rat thymocyte. It is commonly accepted that the A23187 acts as a calcium ionophone, i.e., that it acts as a carrier to facilitate the transport of calcium across biological membranes [2,9]. If this were the case then the antibiotic should be at least as effective in enhancing the uptake of ^{45}Ca from media low in total Ca^{2+} concentration as from media containing higher concentrations of Ca^{2+}. In our studies of the rat thymocyte, however, this proved not to be the case, since A23187 did not increase ^{45}Ca uptake unless substantial concentrations of Ca^{2+} were present in the medium *. One is required to consider, therefore, other mechanisms by which the clearly demonstrated effect of A23187 to increase the uptake of Ca^{2+} in many cellular systems, including the rat thymocyte, is achieved.

We postulate that A23187 binds calcium to form a Ca^{2+}-A23187 complex, which then binds to specific acceptor sites on the membrane. When such sites are sufficiently occupied by the complex, a significant perturbation of the membrane takes place; this permits Ca^{2+} ions to flow freely through the membrane along their electrochemical gradient. According to this model, A23187 does not itself carry calcium through the membrane and is not, therefore, a true ionophore, but rather a quasi-ionophore for calcium.

Support for this hypothesis comes from studies in which lanthanum (La^{3+}) was demonstrated to block the effect of A23187. La^{3+} is thought to block the binding of Ca^{2+} to sites that are involved in the transport of calcium through the membrane [18,19]. Competition with the A23187-

* Lack of effect of A23187 on ^{45}Ca uptake in the Ca^{2+}-free medium was evidently not due to increased cell membrane permeability leading to enhanced ^{45}Ca efflux, since the ability of the cells to exclude [^{3}H]mannitol and trypan blue was essentially the same in Ca^{2+}-free and standard medium. Furthermore, basal uptake of the glucose analogues, 2-deoxyglucose and 3-*O*-methylglucose was found to be the same in both media (unpublished data), whereas their uptake of ^{45}Ca was 2–3-fold higher in the Ca^{2+}-free medium than in the standard medium.

Ca^{2+} complex for these sites could explain the ability of La^{3+} to inhibit both the calcium-dependent effect of A23187 to uncouple oxidative phosphorylation in rat liver mitochondria [2] and its effect to increase DNA synthesis in human peripheral lymphocytes [20].

Whether the mechanism to explain the coordinate effects of A23187 on Ca^{2+} uptake and cell viability in the rat thymocyte, which we herein propose, can be applied to other tissues should be the subject of additional studies.

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References

1 Rasmussen, H. and Goodman, D.B.P. (1972) Physiol. Rev. 57, 421–509
2 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977
3 Wong, D.T., Wilkinson, J.R., Hammill, R.L. and Horng, J.S. (1973) Arch. Biochem. Biophys. 156, 578–585
4 Whitesell, R.R., Johnson, R.A., Tarpley, H.L. and Regan, J.S. (1977) J. Cell Biol. 72, 456–469
5 Reeves, J.P. (1975) J. Biol. Chem. 250, 9428–9430
6 Segal, J. and Ingbar, S.H. (1979) J. Clin. Invest. 63, 507–515
7 Segal, J. and Ingbar, S.H. (1980) Proceedings of the Sixth International Congress of Endocrinology (Cumming, I.A., Funder, J.W. and Mendelsohn, F.A.O., eds.), pp. 405–408, Australian Academy of Sciences, Canberra
8 Zar, J.H. (1974) Biostatistical Analysis, pp. 151–162, Prentice-Hall, Englewood Cliffs, NJ
9 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530
10 Kaiser, N. and Edelman, I.S.E. (1978) Endocrinology 103, 936–942
11 Kaiser, N. and Edelman, I.S.E. 91978) Cancer Res. 38, 3599–3603
12 Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. (1979) Science 206, 700–702
13 Green, W.C., Parker, C.M. and Parker, C.W. (1976) Cell Immunol. 25, 74–89
14 Siraganian, R.P., Kulczycki, A. Jr., Mendoza, G. and Metzger, H. (1975) J. Immunol. 115, 1599–1602
15 Maino, V.C., Green, N.M. and Crumpton, M.J. (1974) Nature 251, 324–327
16 Gerrard, J.M., White, J.G. and Rao, G.H.R. (1974) Am. J. Pathol. 77, 151–159
17 White, J.G. (1974) Am. J. Pathol. 77, 507–518
18 Weiss, G.B. (1974) Annu. Rev. Pharmacol. 14, 343–354
19 Martin, R.B. and Richardson, F.S. (1979) Q. Rev. Biophys. 12, 181–209
20 Luckasen, J.R., White, J.G. and Kersey, J.H. (1974) Proc. Natl. Acad. Sci. USA 71, 5088–5090