Calcium Ionophore A23187 Induces Interleukin 2 Reactivity in Human T Cells

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In the present study the activation of purified human T lymphocytes by the calcium ionophore A23187 was analysed in the light of current concepts of receptor-linked inositol lipid metabolism. It was found that A23187 was only slightly mitogenic, with a narrow optimum at 400–500 nM. The proliferation could be blocked by anti-Tac ascites at 10^3 dilution, suggesting an interleukin-2 (IL-2)-dependent pathway of activation. However, an unexpectedly large proportion of A23187-stimulated cells expressed the IL-2 receptor. Reculturing the cells with exogenous IL-2 after removal of A23187 resulted in strongly enhanced proliferation. Phorbol myristic acetate (PMA) at non-mitogenic concentrations exerted an extremely strong synergistic effect on A23187-induced cell proliferation, which was, again, mediated via an IL-2-dependent pathway. Supernatants of A23187-stimulated T cells did not contain detectable amounts of IL-2. Combination of PMA and A23187 resulted in considerable IL-2 production. It is concluded that A23187 induces the expression of IL-2 receptors without concurrent stimulation of IL-2 production, thus allowing only low levels of proliferation. Addition of exogenous IL-2 or of PMA restores the imbalance between the occurrence of IL-2 and its receptor and results in high rates of proliferation.

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In recent years, much has been added to our understanding of second messenger systems underlying cell-surface receptors. A mechanism that has aroused much interest lately utilizes metabolites of inositol lipids as second messengers. One of these lipids, phosphatidylinositol [4,5]-bisphosphate, is hydrolysed to diacylglycerol (DG) and inositol triphosphate (ITP) upon ligand–receptor interaction. DG activates protein kinase C, a key enzyme in cell activation which is also the target of the tumour-promoting phorbol esters [4]. ITP is released in the cytoplasm and presumably raises free cytosolic calcium (Ca^{2+}), through the opening of calcium ion channels (reviewed in [2]). Recent evidence indicates that the human T lymphocyte uses this bifurcating second messenger mechanism for transmission of signals to the cell nucleus. T-cell mitogens [15] and monoclonal antibodies (MoAb) directed towards the Ti-T3 complex [12, 16, 17] induce a rapid rise in (Ca^{2+}), and a concomitant stimulation of inositol lipid metabolism [6]. In the present report the role of each of the two branches of the messenger system operating in T-cell activation was studied separately. As mentioned above, the DG pathway may be cut short by phorbol myristic acetate (PMA), and the Ca^{2+} influx may be artificially triggered by the ionophore A23187.

MATERIALS AND METHODS

Cell isolation. Peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation on Ficoll-Isopaque (d=1.077 g/cm^3) at 100 g for 20 min and washed twice with Tris-buffered minimal essential medium (MEM, Gibco, USA) plus antibiotics. T cells were purified by two rounds of rosette formation with S-(2-amino-
ethylisothiouronium bromide hydrobromide-treated sheep erythrocytes. The percentage of CD2 (OKT11)-positive cells, as judged by fluorescence microscopy, was routinely ≥98%.

Cell cultures. The T cells were cultured at 10⁶/ml in culture medium: RPMI 1640, supplemented with 10% fetal calf serum (Gibco, USA), l-glutamine, and antibiotics. Proliferation was determined by culturing 2×10⁵ cells in flat-bottomed 96-well microtitre plates; [¹H]thymidine (1 μCi/well; sp. act. 5 mCi/mM, Radiochemical Centre, Amersham, UK) was added 20 h before the end of the culture period. Cells were harvested and the incorporated activity was determined in a Packard liquid scintillation counter. All tests were performed in quadruplicate. In this way the standard deviation never exceeded 10%. A23187 was purchased from Sigma, St Louis, Mo., USA dissolved in dimethylsulphoxide (DMSO) at 10 mM and stored at −20°C. PMA was purchased from Sigma, dissolved in DMSO at 5 mg/ml, and stored at −20°C. Anti-Tac ascites was kindly provided by Dr T. A. Waldmann, Metabolism Branch, NCI-NIH, Bethesda Md., USA. Control ascites was obtained from Balb/c mice inoculated intraperitoneally with the hybridoma fusion partner AG8.

Fluorescence studies. T cells were cultured in 24-well flat-bottomed culture plates in 1 ml at 10⁶/well, harvested and washed twice with MEM. Cells were then suspended in 10 μl of anti-Tac antibody (1:1000 final dilution) and kept at 0°C for 20 min. After a wash with MEM plus 1% bovine serum albumin (BSA), FITC-labelled goat anti-mouse IgG (Becton Dickinson, Mounain University, Calif., USA) was added and the cells were incubated an additional 30 min on ice. After another wash the cells were resuspended in MEM-BSA and analysed on an Ortho-Cytofluorograph. Data analysis was based on reading 10,000 cells per sample.

IL-2 assay. Three times 10⁶ cells of the IL-2-dependent, murine CTL-D cell line were cultured for 24 h in round-bottomed 96-well plates in 100 μl of culture medium with addition of 100 μl of serial dilutions of the supernatants to be assayed. A preparation of recombinant IL-2 (Sandoz, Basle, Switzerland) was used as a reference standard. [¹H]thymidine was added 8 h before the end of the culture period. Incorporated activity was measured as described.

RESULTS

As shown in Fig. 1, A23187 is only moderately mitogenic for human T cells with a narrow optimum at 400–500 mM peaking on day 4. Stimulation of peripheral blood mononuclear cells with phytohaemagglutinin under similar culture conditions routinely results in 20–40 times higher rates of proliferation. Apparently, IL-2 does play an essential role in this modest stimulation by A23187, because the response was completely abrogated by addition of anti-Tac ascites at a final dilution of 10⁻³ (Fig. 2).

![Fig. 2](image-url)  
Fig. 2. T cells were cultured for 4 days in the presence of 0, 400, or 500 nm A23187 in the presence of anti-Tac ascites (+αTac) or of control ascites (−αTac) at 10⁻³ dilution. Proliferation was measured during the last 20 h of culture.

![Fig. 1](image-url)  
Fig. 1. a. Human T cells were cultured for 4 days in the presence of different amounts of A23187. Proliferation was measured during the last 20 h of culture. b. Human T cells were cultured with A23187 at 500 nm. Proliferation was assessed on each day of a 5-day culture.
Fig. 3. T cells were stained with anti-Tac antibody either directly (C) or after 8 h (0), 24 h (1), 48 h (2), 72 h (3), 96 h (4) of culture in the absence (left) or presence (right) of 500 nM A23187. Cell fluorescence was measured on an Ortho cytofluorograph. Each curve depicts the cumulative percentage of fluorescence in arbitrary units for the separate samples. The point of intersection with line (a) denotes the percentage of negative cells in each curve. Thus, culturing cells in the absence of A23187 does not significantly influence Tac expression; in the presence of A23187 the percentage of positive cells increases from 3% in the control of 37% on day 4.

Surprisingly in view of the relatively low responses, we were able to demonstrate a strong expression of the IL-2 receptor by FACS analysis using the anti-Tac antibody (Fig. 3); almost 40% of the cells were positive on day 4 of culture with the ionophore. Because the above set of observations seemed contradictory, we next studied the effect of exogenous IL-2 added to the culture. Addition of IL-2 in the presence of A23187 did not significantly augment cell proliferation (not shown). However, when the cells were washed after 4 days of A23187 stimulation, and subsequently recultured in the presence of recombinant IL-2 (5 units/ml), proliferation was strongly enhanced (Fig. 4). We subsequently investigated a possible synergism between A23187 and PMA with respect to proliferation and IL-2 production. As shown in Fig. 5, otherwise non-mitogenic concentrations of PMA (0.3–1 ng/ml) in combination with A23187 cause an extreme augmentation of proliferation. It is clear from the figure that this effect cannot be explained by simple addition of the mitogenic effects of the separate agents. Although A23187

Fig. 4. T cells were cultured for 4 days in the presence of 0, 300, or 400 nM A23187, washed twice with MEM, and recultured in the presence or absence of recombinant IL2 (5 U/ml). Comparable results were obtained in 4 independent experiments. The results of a representative experiment are depicted.
Fig. 5. Human T cells were cultured for 4 days with different concentrations of A23187 and/or PMA. Proliferation was measured during the last 20 h of culture.

is apparently essential for cell proliferation induced by low amounts of PMA. Higher concentrations of the phorbol ester require less A23187 and in fact are suppressed by addition of the calcium ionophore. Again, proliferation is blocked by anti-Tac ascites (Fig. 6). The hypothesis that the limiting factor in A23187-induced proliferation was a low level of IL-2 production, correctable by addition of PMA, was confirmed by the following experiment. IL-2 production was measured by culturing T cells for 3 days in the presence of different stimuli, washing the cells twice and reculturing them for 1 day in culture medium, after which supernatants were harvested. As shown in Table I, T cells stimulated either by A23187 (250 or 500 nM) or by PMA (1 ng/ml) did not produce any detectable IL-2. However, a combination of 250 nM A23187 and PMA (1 ng/ml) resulted in considerable IL-2 production.

DISCUSSION

Extensive evidence has been gathered on the pivotal role of calcium in early lymphocyte activation (reviewed in [8]). One of the experimental approaches makes use of ionophores, substances that increase the calcium permeability of biological membranes.

In 1974, it was reported that the carboxylic
acid antibiotic A23187, derived from \textit{Streptomyces charteuseus}, stimulated $[^\text{3}P]\text{H}|$thymidine incorporation in pig lymphocytes \cite{10}. Since then many reports have appeared on the mitogenic properties of this calcium ionophore \cite{5,9,13}. In general, it has been found that A23187 induces proliferation at a narrow concentration range and that the proliferation was always of modest magnitude. Recent insights in receptor-linked inositol lipid metabolism have made clear that \textit{a}. ise in intracellular calcium is only one of two important pathways activated after receptor–ligand interaction \cite{2}. This has led us to investigate in more detail the mitogenic properties of A23187 and a possible synergistic effect of this agent with PMA on purified human T cells. PMA activates the second pathway by substituting for DG in the activation of the key enzyme protein kinase C \cite{7}. It was confirmed that A23187 induces relatively little proliferation at optimum concentrations of 400–500 nM, peaking on day 4. The mitogenic effect of the ionophore was abrogated by anti-Tac, suggesting that the observed proliferation of the T cells, however small, depended \textit{a}. Il-2. This is in contradiction to a recent report of Koretzky \textit{et al.} \cite{7}, who found that proliferation induced by A23187 could not be inhibited by anti-Tac antibody. However, these authors used higher dilutions of the antibody. In apparent contrast to the weak mitogenic effect of A23187, it was observed that almost 40\% of the T cells expressed the IL-2 receptor when cultured in the presence of the ionophore. In agreement with the insignificant induction of proliferation, virtually no IL-2 was detectable in supernatants of these cultures. Similar findings were reported by Koretzky \textit{et al.} \cite{7}. The addition of exogenous IL-2 on day 4, after removal of A23187, resulted in strong proliferation within 24 h. Addition of IL-2 together with A23187 at the initiation of the culture did not augment proliferation; an observation which we do not understand as yet. Possibly, the continuous elevation of (Ca$^{++}$), by A23187 interferes with the intracellular signalling of IL-2. Indeed, Birx \textit{et al.} \cite{3} suggest that Ca$^{++}$ is involved in IL-2-induced proliferation.

Beretta \textit{et al.} \cite{1} have reported that A23187 induces responses to T-cell growth factor in mouse lymphocytes, which, in contrast to the present study, were observed during the presence of both IL-2 and A23187.

The combination of otherwise non-mitogenic

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\begin{table}
\centering
\begin{tabular}{|l|c|c|}
\hline
Stimulus & Exp 1 & Exp 2 \\
\hline
A23187, 250 nM & <0.3 & <0.3 \\
A23187, 500 nM & <0.3 & <0.3 \\
PMA, 1 ng/ml & <0.3 & <0.3 \\
A23187, 250 nM+PMA, 1 ng/ml & 8 & 6.5 \\
A23187*, 250 nM+PMA, 1 ng/ml & <0.3 & <0.3 \\
\hline
\end{tabular}
\caption{Interleukin 2 in supernatants of T-cell cultures stimulated with PMA and/or A23187}
\end{table}

The T cell cultures were stimulated for 3 days as indicated, washed twice with MEM and recultured for 24 h in culture medium. Proliferation of the CTL-D cells was not due to passive transfer of PMA and A23187: supernatants of T cells cultured for 2 h instead of 3 days before removal of the stimulating agents did not induce proliferation in the IL-2-dependent cell line.

![Graph](image-url) Fig. 6. T cells were cultured for 4 days with A23187 (at 250 nM) and PMA (1 ng/ml). Control ascites (10$^{-3}$ dilution) or different dilutions of anti-Tac ascites were added on day 0 (end concentrations: 10$^{-3}$, 10$^{-4}$, or 10$^{-5}$). Proliferation was assessed during the last 20 h of culture.
concentrations of PMA with A23187 resulted in a strong enhancement of cell proliferation and a concurrent rise in IL-2 production. Mastro & Smith [11] have reported that bovine lymph node cells proliferate upon stimulation by A23187 followed by a phorbol ester, probably based on a similar mechanism. A synergy has also been reported between calcium ionophores and phorbol ester with respect to murine T lymphocyte proliferation [14]. The production of IL-2 by the Jurkatt cell line is, in a comparable manner, strongly stimulated by the combination of PMA with an agent known to increase (Ca^{++}), such as PHA, OKT3, or A23187 [18].

In conclusion, A23187 induces the expression of functional IL-2 receptors without significantly influencing the production of IL-2. Addition of otherwise non-mitogenic amounts of PMA results in restoration of IL-2 production and leads to cell proliferation.

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