

Activation and proliferation signals in mouse B cells

VII. CALCIUM IONOPHORES ARE NON-MITOGENIC POLYCLONAL B-CELL ACTIVATORS

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Summary. Calcium ionophores cause polyclonal proliferation of lymphocytes from man, rabbit and pig, but are not mitogenic for mouse T or B lymphocytes. We show here that two Ca^{2+} ionophores (A23187 and ionomycin) nonetheless activate a substantial proportion of mouse B lymphocytes at concentrations which effectively inhibit DNA synthesis induced by conventional mitogens, such as anti-immunoglobulin antibodies. Activation of B cells was detected by (i) increased expression of Ia antigen after 24 hr culture with ionophores, and (ii) the accelerated onset of DNA synthesis in B cells primed with ionophores for 24 hr, washed and then rechallenged with anti-Ig. Unlike anti-Ig, the ionophores did not induce either the breakdown of inositol phospholipids, or RNA synthesis in B cells. Finally, activation of B cells by ionophores is highly susceptible to inhibition by cyclosporine. These results therefore suggest that elevation of intracellular Ca^{2+} induced by these ionophores is sufficient to cause B cells to leave G_0 , but not to enter the G_1 phase of the cell cycle. Clearly, additional signals are required for B cells to progress further into cycle and eventually become committed to DNA synthesis.

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INTRODUCTION

The role of Ca^{2+} in mitogen-induced lymphocyte proliferation has been the subject of some controversy (reviewed by Lichtman, Segal & Lichtman, 1983). It seems clear that lymphocytes require extracellular Ca^{2+} to proliferate in response to lectins (Alford, 1970; Whitney & Sutherland, 1972). However, since DNA synthesis only commences after some 48–72 hr, these findings tell us little about the stage of the response at which Ca^{2+} is required, and, more specifically, if Ca^{2+} is involved in receptor signalling by antigens and/or growth factors. Many receptor systems in non-lymphoid cells appear to employ Ca^{2+} as a second messenger for transducing extracellular signals into intracellular responses, and within recent years it has emerged that some (but not all) polyclonal lymphocyte activators also cause a rapid increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Tsien, Pozzan & Rink, 1982; Bijsterbosch *et al.*, 1985).

If a rise in $[\text{Ca}^{2+}]_i$ levels can lead to lymphocyte activation, then agents which cause entry of Ca^{2+} into the cells (i.e. Ca^{2+} ionophores) might act as lymphocyte mitogens. This is indeed the case with lymphocytes from certain species. Thus, the carboxylic Ca^{2+} ionophore A23187 is mitogenic for pig, rabbit and human lymphocytes (Maino, Green & Crumpton, 1974; Resch, Bouillon & Gemsa, 1978; Hovi, Allinson & Williams, 1976; Luckasen, White & Kersey, 1974).

However, A23187 is not mitogenic for either T or B lymphocytes from the mouse (Maino, Green & Crumpton, 1974; Rosenstreich & Blumenthal, 1977; Beretta *et al.*, 1984).

It has recently become clear that stimulation of resting lymphocytes by mitogens can be operationally divided into two stages with differing signalling requirements: (i) activation, or exit from G_0 , and (ii) commitment to DNA synthesis. This concept is supported by the recent description of a variety of non-mitogenic polyclonal activators for mouse B cells, which activate these cells but do not induce DNA synthesis (Hawrylowicz, Keeler & Klaus, 1984; Hawrylowicz & Klaus, 1984; Klaus *et al.*, 1984; Monroe & Cambier, 1984). We therefore wished to study if Ca^{2+} ionophores have similar effects on mouse B cells. The present results indicate that this is so.

MATERIALS AND METHODS

Experimental animals

Male CBA/Ca and (CBA \times C57BL/6) F_1 mice bred under specific pathogen-free conditions at NIMR were used as sources of splenic B cells at 3–4 months of age.

Reagents

The preparation of affinity-purified $F(ab')_2$ fragments of rabbit anti-mouse Fab antibodies (henceforth called anti-Ig) and of fluorescein isothiocyanate (FITC)-coupled monoclonal anti-I-A^k antibody have been described (Klaus *et al.*, 1984). Lipopolysaccharide (LPS: *E. coli* 055:B5W) was from Difco (Detroit, MI), A23187 and phorbol myristic acetate (PMA) were from Sigma (Poole, Dorset) and ionomycin was from Cambridge Bioscience (Cambridge) [3H]myo-inositol (specific activity 15 Ci/mmol), [3H]thymidine ([3H]TdR, specific activity 5 Ci/mmol), and [3H]uridine ([3H]UrD, specific activity 2–5 Ci/mmol) were from Amersham International, Amersham, Bucks. Cyclosporine (CS) was kindly donated by Dr J. F. Borel, Sandoz Ltd, Basle, Switzerland.

Culture system

This has been described in detail elsewhere (Hawrylowicz *et al.*, 1984; Klaus *et al.*, 1984). In some experiments, small dense B cells were prepared on discontinuous gradients of Percoll (Pharmacia, Uppsala, Sweden) and additionally filtered through two consecutive columns of Sephadex G10 to remove

adherent cells as described by Klaus *et al.* (1984). The effluent cells were at least 90% surface Ig-positive by immunofluorescent analysis.

Assays for B-cell activation

These were all done as described previously: analysis of Ia-antigen expression using the Fluorescence Activated Cell Sorter (FACS) (Klaus *et al.*, 1984), entry into cell cycle in two-stage priming cultures (Klaus *et al.*, 1984), and measurements of breakdown of phosphatidylinositol 1,4 bis-phosphate (PIP₂) as described by Bijsterbosch *et al.* (1985).

RESULTS

Ca^{2+} ionophores are not mitogenic, but inhibit B-cell proliferation

In the experiment summarized in Fig. 1, B cells were cultured with 250 ng/ml A23187, 1 μ g/ml ionomycin,

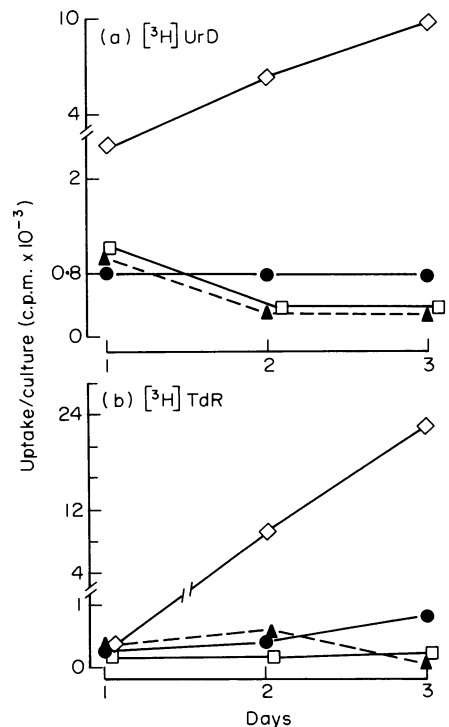


Figure 1. Effects of Ca^{2+} ionophores on RNA and DNA synthesis in B cells. B cells were cultured with medium alone (●), with 50 μ g/ml anti-Ig (◇), 250 ng/ml A23187 (▲), or with 1 μ g/ml ionomycin (□). On Days 1, 2 and 3, cohorts of these cultures were labelled with (a) [3H]UrD, or (b) [3H]TdR, and these were harvested 4 hr later.

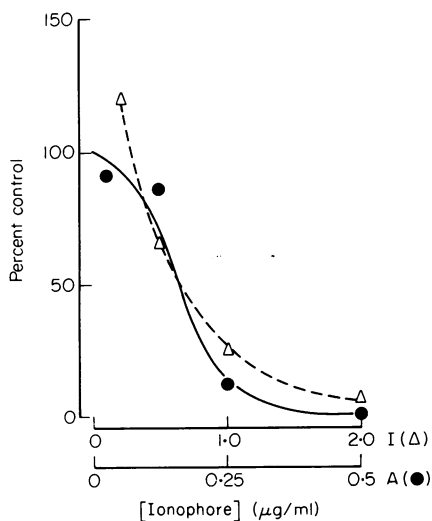


Figure 2. Effects of Ca²⁺ ionophores on B-cell proliferation induced by anti-Ig. Cultures were stimulated with 50 µg/ml anti-Ig, either alone, or in the presence of the indicated concentrations of A23187 (A), or ionomycin (I). [³H]TdR uptakes were assayed on Day 3. Control responses: background 730 c.p.m., anti-Ig alone 23,600 c.p.m.

or 50 µg/ml anti-Ig. The kinetics of [³H]UrD and [³H]TdR uptakes were followed for 3 days. It is clear that neither ionophore induced significant increases in [³H]UrD incorporation in B cells, and eventually both suppressed [³H]UrD and [³H]TdR uptakes to below background levels. Similar results were obtained over a broad range of ionophore concentrations, ranging from 31 to 500 ng/ml of A23187, and 62 to 2000 ng/ml ionomycin (data not shown). Anti-Ig, on the other hand, gave the expected increases in both RNA and DNA synthesis.

In a further experiment, B cells were cultured with a mitogenic concentration of anti-Ig in the presence of varying concentrations of either ionophore. As shown in Fig. 2, both ionophores caused dose-dependent suppression of DNA synthesis: concentrations causing 50% inhibition (IC₅₀) were 650 ng/ml for A23187, and 160 ng/ml for ionomycin.

Ca²⁺ ionophores induce B cell activation

Effects on Ia antigen expression. It is well-established that the levels of Ia antigens expressed by B cells increase substantially within 12–20 hr after stimulation by a wide variety of polyclonal activators (Mond *et al.*, 1981). In the experiments summarized in Fig. 3,

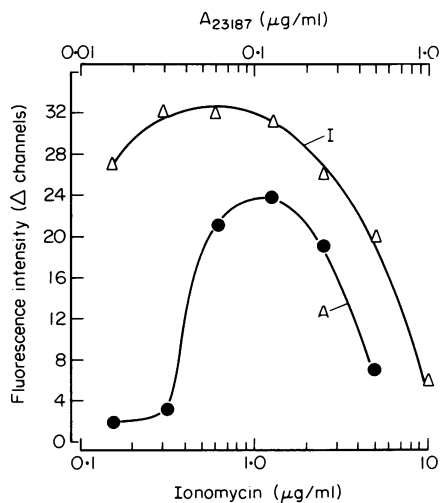


Figure 3. Effects of Ca²⁺ ionophores on levels of I-A antigen on B cells. Cells from CBA/Ca mice were cultured for 24 hr with the indicated concentrations of A23187 (A: Exp. 1), or ionomycin (I: Exp. 2). The cells were then stained with FITC-anti-I-A^k, and analysed on the FACS. Data are presented as differences (Δ) between median fluorescence intensities of stimulated and control cultures, derived from computer-generated histograms, and are given in numbers of channels: 51 channels are equivalent to 1 log difference in intensity. Ten µg/ml anti-Ig gave median increases in staining of 39 channels in Exp. 1, and 42 channels in Exp. 2.

B cells from CBA/Ca mice were cultured for 24 hr with various concentrations of A23187 or ionomycin, and the cultures were then stained with FITC-anti-I-A^k prior to analysis on the FACS.

Both ionophores caused significant dose-related increases in the levels of I-A expressed by B cells, although the effects were somewhat less dramatic than the response induced by an optimal concentration of anti-Ig. The response to A23187 displayed a fairly sharp optimum at approximately 100 ng/ml, while ionomycin gave an essentially plateau response between 100 and 1000 ng/ml. As has been noted previously with other polyclonal activators, effectively all B cells appeared to respond to the ionophores (data not shown).

Priming for accelerated DNA synthesis to anti-Ig.

Exit of B cells from G₀ into cycle can be demonstrated by culturing (priming) the cells for 24 hr with potentially mitogenic, or non-mitogenic activators, and then reculturing the washed cells with a mitogenic concentration of anti-Ig (Klaus *et al.*, 1984). Priming is

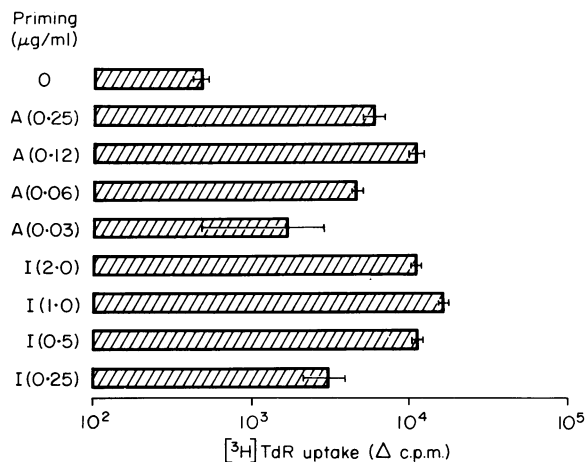


Figure 4. B-cell priming by Ca^{2+} ionophores. Cultures were incubated with the indicated concentration of A23187 (A), or ionomycin (I). After 24 hr, these cultures were washed, counted, and recultured with or without 50 $\mu\text{g}/\text{ml}$ anti-Ig. The readout cultures were assayed for DNA synthesis after 24 hr. Results are expressed as means \pm SEM, with backgrounds subtracted (Δ c.p.m.). Cultures of B cells primed with 10 $\mu\text{g}/\text{ml}$ anti-Ig gave a response of 28,900 c.p.m. in this experiment.

manifested by the earlier onset of DNA synthesis in response to restimulation.

Such an experiment using the two Ca^{2+} ionophores is summarized in Fig. 4. B cells were primed for 24 hr with 30–250 ng/ml A23187, or with 0.25–2.0 $\mu\text{g}/\text{ml}$ ionomycin. After restimulation with anti-Ig, ^3H TdR uptakes were assayed 24 or 48 hr (not shown) later. After 24 hr, unprimed cells gave a barely detectable response, since B cells require some 30 hr continuous exposure to anti-Ig to commence DNA synthesis. It is clear that both ionophores induced dose-related prim-

ing of B cells: optimal responses were elicited by 125 ng/ml A23187, and by 1.0 $\mu\text{g}/\text{ml}$ ionomycin. However, 10 $\mu\text{g}/\text{ml}$ anti-Ig induced substantially greater priming. Within the dose ranges used, neither ionophore was cytotoxic, as judged by cell recoveries from the priming cultures (data not shown). Finally, similar results were obtained if primed B cells were challenged with LPS, rather than with anti-Ig (data not shown).

In a further experiment, small dense B cells depleted of adherent cells were cultured as above. These cells, which were >90% Ig^+ , were effectively activated by

Table 1. Priming of adherent cell-depleted B cells by ionomycin

Priming stimulus* ($\mu\text{g}/\text{ml}$)	Response to anti-Ig† (c.p.m./culture)
None	6570 \pm 440
Ionomycin (1.0)	11,850 \pm 130
Ionomycin (0.5)	15,590 \pm 1210
Ionomycin (0.25)	13,320 \pm 500
Anti-Ig (10)	47,300 \pm 470

*Small dense B cells prepared on Percoll gradients were passed through Sephadex G10 and cultured for 24 hr with the indicated additions.

† After washing, these cells were recultured with, or without, 50 $\mu\text{g}/\text{ml}$ anti-Ig: ^3H TdR uptakes in these readout cultures were determined 48 hr later. Data are means \pm SEM of three cultures/group, with backgrounds (270–560 c.p.m.) subtracted.

ionomycin, or by anti-Ig (Table 1). We therefore conclude that Ca²⁺ ionophores activate B cells, and that (operationally) this does not appear to require substantial numbers of accessory cells.

Ca²⁺ ionophores do not induce phosphoinositide breakdown

It has recently become clear that anti-Ig induces the breakdown of cell membrane-associated PIP₂ in B cells, and that this is associated with an elevation of [Ca²⁺]_i (Coggeshall & Cambier, 1984; Bijsterbosch *et al.*, 1985). These early biochemical changes have been implicated in mediating a wide variety of cellular responses, including the induction of cell growth (Berridge, 1984).

In the experiment summarized in Table 2, adherent

Table 2. Effects of Ca²⁺ ionophores or anti-Ig on phosphoinositide degradation in B cells

Stimulus* ($\mu\text{g/ml}$)	% radiolabel in inositol phosphates†
Medium	1.8 \pm 0.1
A23187 (0.125)	1.7 \pm 0.2
Ionomycin (1.0)	1.4 \pm 0.2
Anti-Ig (50)	18.1 \pm 1.6

* Adherent cell-depleted [³H]inositol-labelled B cells (90–94% Ig-positive) were incubated with the indicated additions for 1 hr at 37° in the presence of 5 mM LiCl.

† Percentage of cell-associated radiolabel (30,000 d.p.m. on average) in combined inositol phosphate fraction. Means \pm SEM of four replicates from two separate experiments.

cell-depleted B cells were labelled with [³H]inositol and were incubated for 1 hr with optimally activating concentrations of A23187, ionomycin or anti-Ig. Breakdown of PIP₂ was assayed by extracting the mixture of [³H]inositol phosphates (Bijsterbosch *et al.*, 1985). In agreement with earlier results, anti-Ig induced a substantial accumulation of inositol phosphates. However, neither ionophore elicited a detectable response.

Activation by ionophores is inhibited by cyclosporine

Previous work has shown that activation of mouse B cells by anti-Ig is highly susceptible to inhibition by the

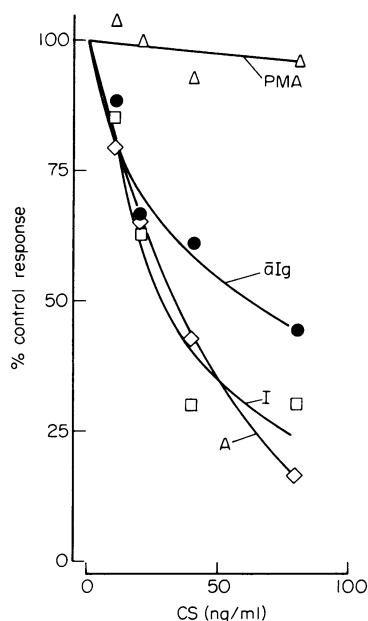


Figure 5. Effects of cyclosporine on increases in I-A antigen expression induced by various B-cell activators. CBA/Ca B cells were cultured for 24 hr with anti-Ig (10 $\mu\text{g/ml}$), PMA (0.01 $\mu\text{g/ml}$), A23187 (A: 0.125 $\mu\text{g/ml}$), or ionomycin (I: 0.5 $\mu\text{g/ml}$), in the presence or absence of varying concentrations of CS (dissolved in Tween:alcohol). The cultures were then stained and analysed as described in Fig. 3. Data are presented as percentages of control responses (Δ medians) which were as follows: anti-Ig, 33; PMA, 29; A23187, 30; ionomycin, 27.

immunosuppressive agent CS, while stimulation by LPS is drug resistant (Dongworth & Klaus, 1982). This suggested the existence of CS-sensitive and -resistant activation pathways in B cells (Klaus & Hawrylowicz, 1984). We therefore studied the effects of CS on ionophore-induced B-cell activation. This was done by measuring the effects of CS on increases in Ia antigen expression induced by various activators (Fig. 5). In agreement with our earlier results, the response to PMA was unaffected by 80 ng/ml CS, while that to anti-Ig was 50% inhibited by 60 ng/ml. The responses to both ionophores were also markedly inhibited, with IC₅₀ values of around 30 ng/ml.

DISCUSSION

The results of this study confirm that Ca²⁺ ionophores are not mitogenic for mouse B lymphocytes (Rosen-

streich & Blumenthal, 1977), but instead inhibit DNA synthesis induced by conventional mitogens (Maino *et al.*, 1974), presumably as a result of long-term toxicity. However, it is clear that both ionomycin and A23187 activate mouse B cells: both ionophores caused substantial increases in the levels of I-A antigen expressed by these cells (Fig. 3), and also primed B cells to give enhanced (i.e. accelerated) DNA synthetic responses after restimulation with anti-Ig (Fig. 4). We have shown previously that priming reflects entry of resting (G_0) cells into cell cycle (Klaus *et al.*, 1984). However, neither ionophore induced detectable increases in RNA synthesis in B cells (Fig. 1), and thus do not appear to drive the cells into classical G_1 .

We therefore conclude that Ca^{2+} ionophores represent further examples of non-mitogenic polyclonal B-cell activators. The agents induce resting cells to leave G_0 and appear to drive them into a transitional activated state (termed G_{IT} by Darzynkiewicz, Traganos & Melamed, 1980), between deep quiescence and G_1 proper. Other examples of this type of activator are PMA, concanavalin A, and the intact [rather than the $F(ab')_2$] form of anti-Ig (Hawrylowicz *et al.*, 1984; Klaus *et al.*, 1984; Hawrylowicz & Klaus, 1984; Monroe & Cambier, 1984). The physiological significance of this transitional activated state in B cells is still uncertain, although it is possible that the above agents mimic the effects of specific antigens on these cells (Monroe & Cambier, 1983).

Recent evidence suggests that A23187 may have similar effects on mouse T lymphocytes, where it has been shown to induce the expression of receptors for interleukin-2, but only stimulates weak or insignificant DNA synthesis (Beretta *et al.*, 1984). It is likely that these activating effects in both T and B cells reflect ionophore-induced Ca^{2+} entry into the cells. In line with this, increases in Ia antigen expression on B cells are abrogated by chelating extracellular Ca^{2+} (data not shown). Although Freedman *et al.*, (1981) concluded that A23187 did not induce $^{45}Ca^{2+}$ uptake into mouse B cells, we have shown by direct measurements of $[Ca^{2+}]_i$ levels using the Ca^{2+} indicator quin2 that both A23187 and ionomycin do cause Ca^{2+} influx in these cells, as expected (data not shown).

The present results therefore strongly suggest that raising $[Ca^{2+}]_i$ levels in mouse B lymphocytes is sufficient to activate these cells, but that additional signals are required to cause further cell cycle progression and commitment to DNA synthesis. It has recently become clear that surface Ig receptors belong to the widely distributed group of receptor systems

which cause the breakdown of PIP_2 in the cell membrane (Coggeshall & Cambier, 1984; Bijsterbosch *et al.*, 1985). One of the primary breakdown products (inositol 1,4,5 tris-phosphate) is believed to be involved in Ca^{2+} mobilization, while the other (1,2 diacylglycerol) is known to be a cofactor of protein kinase C (reviewed by Berridge, 1984; Nishizuka, 1984). Both these products therefore have potential second messenger functions in inducing appropriate cellular responses. However, there is evidence from several cell types to suggest that optimal signalling requires the generation of both arms of the response (Nishizuka, 1984).

The data available for lymphocytes are consistent with this dual signal hypothesis. As expected, Ca^{2+} ionophores do not cause breakdown of PIP_2 in B cells (Table 2), thus confirming earlier results with thymocytes (Taylor *et al.*, 1984). Instead, these agents presumably bypass this normally receptor-mediated event and raise $[Ca^{2+}]_i$ passively. PMA is known to substitute for diacylglycerol in activating protein kinase C (Nishizuka, 1984), and this agent also activates mouse B cells without inducing DNA synthesis (Hawrylowicz *et al.*, 1984; Monroe & Cambier, 1984). Ca^{2+} ionophores plus PMA should therefore be able to mimic the two signals resulting from ligation of antigen receptors by appropriate reagents, and in consequence induce lymphocyte proliferation. Recent data confirm that this is the case for both mouse T cells (Truneh *et al.*, 1985) and B cells (G. G. B. Klaus *et al.*, submitted).

The classical B-cell mitogen, LPS, does not cause breakdown of PIP_2 or raise $[Ca^{2+}]_i$ in these cells (Bijsterbosch *et al.*, 1985). This suggests that there is a pathway of B-cell activation which bypasses these early biochemical steps. Consistent with this are observations showing that cyclosporine inhibits B-cell activation by anti-Ig, but not by LPS or PMA (Dongworth & Klaus, 1982; Klaus & Hawrylowicz, 1984). We now demonstrate that activation of B cells by Ca^{2+} ionophores is also very susceptible to inhibition by CS (Fig. 5), thereby confirming earlier results with pig lymphocytes (Kay, Benzie & Borghetti, 1983). These findings strengthen the hypothesis that CS interferes with some event(s) along the Ca^{2+} -dependent pathway of lymphocyte activation. The nature of the lesion induced by CS is still unknown. However, it must be distal to mitogen-induced PIP_2 degradation and Ca^{2+} mobilization, since neither of these early effects is blocked by the drug (Metcalf, 1984; Bijsterbosch & Klaus, 1985).

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