Limits to the early increase in free cytoplasmic calcium concentration during the mitogenic stimulation of lymphocytes

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Three aspects of the calcium hypothesis we have proposed previously [Metcalf, Pozzan, Smith & Hesketh (1980) *Biochem. Soc. Symp.* **45**, 1–26] for the control of mitogenic stimulation of lymphocytes are examined in studies on the mitogenic action of the Ca^{2+} ionophore A23187 and its effect on cap formation. (1) Pig lymphocytes that were mitogenically stimulated by continuous incubation with ^3H -labelled A23187 for 48 h contained between 3 and 15 amol of ionophore per cell. Lymphocytes exposed to ^3H -labelled A23187 for 2 h before washing the cells and resuspending them in ionophore-free medium were only stimulated mitogenically at 48 h if the residual ionophore associated with the cells after washing was in the concentration range 3–15 amol per cell. When the cells were washed repeatedly after 2 h incubation with ionophore to reduce the cell-associated ionophore below the critical concentration range, no mitogenic stimulation occurred as a result of short-term exposure to any ionophore concentration. Re-addition of ionophore to within the indicated range of cell-associated concentrations restored mitogenic stimulation at 48 h. We conclude that large, short-term Ca^{2+} fluxes into the cells induced by the ionophore cannot generate a mitogenic signal that commits the cells to enter the cell cycle. (2) Further experiments with the ionophore showed that detectable mitogenic stimulation at 48 h required a minimum of 3 h exposure to optimal ionophore concentrations, and that maximal stimulation required at least 20 h exposure. This is consistent with the view that a prolonged increase in the free cytoplasmic calcium concentration is required to stimulate the maximum proportion of the cells into the cell cycle. (3) Mouse splenic lymphocytes treated for short periods with very high ionophore concentrations (30 μM) in the presence of various external Ca^{2+} concentrations showed significant inhibition of cap formation of surface immunoglobulin receptors in the range 1–10 μM - Ca^{2+} in normal or depolarizing medium. We conclude that mitogens at optimal concentrations for the stimulation of lymphocytes do not cause any early increase in the free cytoplasmic Ca^{2+} concentration above 10 μM .

We have recently proposed a calcium hypothesis for the stimulation of resting lymphocytes into the cell cycle by mitogens that has three main features (Metcalf *et al.*, 1980). It proposes that the primary mitogenic signal is an increase in the free cytoplasmic calcium concentration in the range below 1 μM , and that free calcium concentrations in the cytoplasm substantially above this range inhibit

mitogenic stimulation and are eventually cytotoxic. The third feature of the hypothesis is that the free cytoplasmic calcium concentration must remain at the enhanced level appropriate for passage into the cell cycle for a time comparable with the period before increased DNA synthesis can be detected (about 24 h), to induce the maximum proportion of cells to enter the cell cycle: lowering the free calcium concentration in the cytoplasm at earlier times progressively reduces the proportion of committed cells.

The basis for this working hypothesis came from several lines of evidence. The action of the calcium ionophores A23187 and ionomycin as mitogens on

Abbreviations used: FITC-RaMIg, fluorescein isothiocyanate-labelled rabbit anti-(mouse immunoglobulin).

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pig and human lymphocytes (Maino *et al.*, 1974; Luckasen *et al.*, 1974; Lyall *et al.*, 1980) suggested that calcium fluxes might be directly involved in the mechanism of mitogenic stimulation. Our work on $^{45}\text{Ca}^{2+}$ fluxes in pig and mouse lymphocytes had indicated that any obligatory increase in total cell calcium concentration under conditions where the ionophore (or concanavalin A) acted as a mitogen was less than $50\mu\text{M}$, and that larger prolonged increases in the total cell calcium concentration inhibited mitogenic stimulation (Hesketh *et al.*, 1977). In preliminary studies (Metcalf *et al.*, 1980) it was shown that large, short-term increases in intracellular $[\text{Ca}^{2+}]$ induced by A23187 were not mitogenic, and that the inhibitory effect of the ionophore on cap formation could be used to refine the limit to any increase in free cytoplasmic $[\text{Ca}^{2+}]$ that may occur as an early event in mitogenic stimulation. Here we give a full experimental account of these latter studies and assess the current status of the hypothesis in relation to recent data.

Materials and methods

Cell preparations

Suspensions of pig lymphocytes were prepared from lymph nodes removed from the mesentery of animals within 45 min of slaughter. The nodes were disrupted by pressing through a stainless-steel strainer to release the cells into a medium of RPMI 1640 supplemented with 0.2% NaHCO_3 , 20 mM-Tris/HCl, 0.05 mg of gentamycin/ml and either 2 mM-glutamine or 10% foetal-calf serum (Flow Labs.), at pH 7.2. Mouse spleen cells were similarly prepared from BALB/c mice. Cell suspensions were then layered on to Ficoll/Hypaque and centrifuged for 20 min at 525 g. The interfacial layer of cells was removed, suspended in medium, washed twice by centrifugation and finally suspended in medium at the concentrations appropriate for capping or mitogenic stimulation experiments.

Mitogenic stimulation

Cells were incubated in 10 ml glass tubes at 6×10^6 cells/1 ml of medium. The appropriate concentration of ^3H -labelled A23187 or unlabelled A23187 was added from a dilution in medium of a stock solution of the ionophore in dimethyl sulphoxide (1 mg/ml). In the washing procedure to remove ionophore and reduce its concentration the cells were diluted with 9 ml of medium, centrifuged for 5 min at 525 g and, after removing the supernatant solution, the cell pellet was resuspended in 1 ml of fresh medium pre-warmed to 37°C . In experiments using ^3H -labelled A23187 the cell-associated ionophore was estimated both by depletion of radioactivity from the supernatant solution and from the radioactivity of the cell pellet in

replicate cultures. A23187 (Eli Lilly) was labelled by ^3H exchange using trifluoro ^3H acetic acid in chloroform and purified by chromatography on a silica column. The specific radioactivity was 50 Ci/mol.

The cells were cultured in a gassed incubator (CO_2/air , 1:19; 37°C) for 42 h before being pulsed for 6 h with $1.5\mu\text{Ci}$ of ^3H thymidine (Amersham International) per 1 ml of culture. Cells were harvested by filtration on to Whatman GF/A glass-fibre filters and assayed for radioactivity in a liquid-scintillation counter.

Capping assays

Mouse spleen cells were prepared as described above in a medium of inorganic salts of RPMI 1640 (120 mM-NaCl/5 mM-KCl/0.5 mM-MgSO $_4$ ·7H $_2$ O/5 mM-Na $_2$ HPO $_4$) or in 10 mM-NaCl/120 mM-KCl/0.5 mM-MgSO $_4$ ·7H $_2$ O/5 mM-Na $_2$ HPO $_4$, each supplemented with 11 mM-glucose and 20 mM-Tris/HCl, pH 7.3. The Ca^{2+} concentration in these solutions (approx. $9\mu\text{M}$) was measured by using a Perkin-Elmer 380 atomic-absorption spectrophotometer. The free Ca^{2+} concentrations indicated in Fig. 4 were obtained either by addition of CaCl_2 or by the addition of 10 mM-EGTA buffer and the required amount of CaCl_2 calculated by an iterative computer program based on the method of Fabiato & Fabiato (1979). FITC-RaMIg (Miles Yeda) was extensively dialysed against phosphate buffer to remove merthiolate before addition at $250\mu\text{g/ml}$ to lymphocyte suspensions at 10^7 cells/ml. The suspension was incubated for 10 min at 37°C before dilution with 1 ml of ice-cold phosphate-buffered saline, followed by centrifugation (5 s, 14 000 g) and suspension of the pellet in a 1% solution of paraformaldehyde in phosphate-buffered saline. Cells were examined in a Leitz inverted-stage fluorescence microscope and capped cells were scored as the percentage of fluorescent-labelled cells (200 per sample) on which the label was restricted to less than one-half of the cell surface. In capping experiments, the appropriate concentration of A23187 was added from a suspension of (dioleoyl)phosphatidylcholine (dioleoylglycerophosphocholine) liposomes.

Results

Short-term exposure to ionophore

Pig lymphocytes were treated with high concentrations of ^3H -labelled A23187 for 2 h periods to determine whether a short, large Ca^{2+} pulse is sufficient to stimulate the cells. Cells treated continuously with the ionophore for 48 h in the presence of 10% foetal-calf serum show optimal mitogenic stimulation in the range $0.7\text{--}1.0\mu\text{M}$ ^3H -labelled A23187 (Fig. 1). (Fig. 3 also includes data points for continuous 48 h incubation under the same condi-

Fig. 1. Uptake of $[^3\text{H}]$ thymidine between 42 and 48 h by 6×10^6 pig lymphocytes continuously stimulated in the presence of 10% foetal-calf serum (FCS; Δ), washed after 2 h and resuspended in ionophore-free medium with 10% foetal-calf serum ($\square$) or stimulated continuously in the absence of foetal-calf serum ($\blacklozenge$).

Cells washed twice (at 2 and 3 h) are not mitogenically stimulated ($\circ$) unless A23187 is subsequently added back to the cultures ($\bullet$).

Fig. 2. Uptake of $[^3\text{H}]$ thymidine between 42 and 48 h as a function of ^3H -labelled A23187 associated with cells. Cells were treated as described in the legend to Fig. 1. Symbols: Δ , continuous A23187 + 10% foetal-calf serum; $\blacklozenge$, continuous A23187 in the absence of foetal-calf serum; $\square$, washed after 2 h and suspended in ionophore-free medium; $\circ$, washed twice at 2 h and 3 h; $\bullet$, washed twice at 2 and 3 h, with A23187 re-addition at 3 h. Cell samples were finally centrifuged and the pellets were counted for ^3H -labelled A23187 radioactivity at $3\frac{1}{2}$ h.

tions with 10% serum using non-radioactive ionophore: of the four concentrations used, $1.3\mu\text{M}$ -A23187 gave the largest stimulation.) When the cells were washed once after incubation for 2 h with ionophore and suspended in fresh medium containing 10% serum with no further addition of A23187, the optimal concentration for mitogenic stimulation for the 2 h incubation was $13\mu\text{M}$. However, if the cells were washed a second time at 3 h and suspended in medium with 10% serum, no mitogenic stimulation occurred. These cells could be stimulated mitogenically by adding back A23187, with an optimal concentration of additional ionophore of approximately $0.3\mu\text{M}$. The $[^3\text{H}]$ thymidine incorporation at 42–48 h was between 50 and 100% of the normal maximal value (Fig. 1). Thus the loss of mitogenic stimulation in the cells washed twice (at 2 and 3 h) was mainly due to the removal of ionophore, rather than repeated manipulation of the cells by washing.

Similar results were obtained for cells stimulated by ionophore in the absence of serum. The optimal concentration of ionophore for mitogenic stimulation after 48 h continuous incubation was $0.1\mu\text{M}$ (Fig. 1), which is substantially lower than in the presence of serum because serum buffers the free ionophore concentration as described previously (Hesketh *et al.*, 1977). For cells in the absence of serum that were washed after 2 h incubation with ionophore, the optimal concentration for mitogenic stimulation was $0.6\mu\text{M}$ (results not shown). The mitogenic stimulation obtained in this experiment

was unaffected by the presence of 2 mM-EGTA during the 2 h incubation with ionophore, provided that the cells were resuspended from 2–48 h in medium containing the normal Ca^{2+} concentration (0.43mM) after washing. This indicates that the entry of Ca^{2+} into the cells mediated by supra-optimal ionophore concentrations for 2 h is irrelevant to their subsequent mitogenic stimulation. Cells incubated for 2 h at concentrations significantly lower than $0.6\mu\text{M}$ -A23187 before washing were only stimulated if ionophore was added back after the washing step.

When the amount of $[^3\text{H}]$ A23187 associated with the cells in the above experiments was measured 1 h after washing in medium (to allow re-equilibration of the residual ionophore), it was found that significant stimulation occurred only when the amount of cell-associated ionophore was within the range 3–15 amol per cell (Fig. 2), irrespective of the presence or absence of serum, or of the prior washing of the cells to remove A23187. Thus, in the experiments in which cells were washed a second time at 3 h and no significant mitogenic stimulation was subsequently observed (Fig. 1), the amount of residual ionophore had been reduced to less than 2 amol per cell (Fig. 2). Re-addition of $[^3\text{H}]$ A23187 to the twice-washed cells caused mitogenic stimulation within the indicated range of cell-associated ionophore concentration (Fig. 2). We conclude that the lymphocytes require prolonged exposure to a defined concentration range of bound ionophore for significant stimulation to occur.

Fig. 3. $[^3\text{H}]$ Thymidine incorporated between 42 and 48 h by pig cells incubated in the presence of various concentrations of A23187 ($0.8\ \mu\text{M}$, $\circ$; $1.3\ \mu\text{M}$, $\blacktriangle$; $3.8\ \mu\text{M}$, $\blacksquare$; $9.6\ \mu\text{M}$, $\triangle$) and 10% foetal-calf serum. At the times shown cells were washed and re-suspended in ionophore-free medium + foetal-calf serum.

Toxic effects of prolonged ionophore exposure

The requirement for prolonged exposure with ionophore for mitogenic stimulation is further illustrated in Fig. 3. The cells were incubated with a range of ionophore concentrations in the presence of 10% serum and washed at different times before suspending in ionophore-free medium with 10% serum. It can be seen that continuous incubation with $1.3\ \mu\text{M}$ -A23187 causes relatively high stimulation measured at 42–48 h. Furthermore the ionophore must interact with the cells for at least 3 h to induce significant stimulation at 48 h and for at least 12 h to cause the maximum stimulation obtainable with $1.3\ \mu\text{M}$ -A23187. Exposure to $3.8\ \mu\text{M}$ -A23187 for 18–30 h stimulated the maximal number of cells, estimated to be 70–80% of the total population (Hesketh *et al.*, 1977), in agreement with the previous observations of Maino *et al.* (1974). Removal of most of the ionophore by washing at 24 h prevented the decline in the response measured at 42–48 h (Fig. 3), which indicates that a substantial proportion of the cells activated by ionophore at 24 h are sensitive to prolonged interaction with the ionophore thereafter. At supraoptimal ionophore concentrations ($9.5\ \mu\text{M}$ in Fig. 3), the ionophore was toxic after exposure to the cells for 48 h. However, if the duration of the exposure to $9.5\ \mu\text{M}$ -ionophore did not exceed 24 h, a small subpopulation of the cells remained viable and were subsequently stimulated by 48 h. Even this elevated ionophore concentration was unable to stimulate a subpopulation of the cells unless present for at least 12 h.

The ability to 'rescue' cells committed to the cell cycle from the toxic effects of prolonged exposure to

the ionophore may be demonstrated by an alternative method in which the cells are initially incubated with ionophore in serum-free medium. The addition of 10% serum then substantially reduces the cell-associated ionophore concentration by acting as a buffer for the ionophore; results very similar to those shown in Fig. 3 were obtained when serum was added at the appropriate time instead of washing the cells.

The requirement for extracellular Ca^{2+} to inhibit capping with A23187

It has been shown previously by Schreiner & Unanue (1976) and confirmed by us that capping occurs in media without free calcium, which implies that the entry of calcium into the cells across the plasma membrane is not necessary for cap formation. On the other hand, it was also shown that ionophore concentrations that cause a detectable increase in calcium uptake will both block cap formation and cause the dispersal of surface immunoglobulin caps on B-lymphocytes in normal media containing calcium (Schreiner & Unanue, 1976). These observations offer an indirect method of setting an upper limit to the concentration of free calcium in the cytoplasm at optimal concentrations of mitogens for stimulation. If we use a very high concentration of ionophore, which blocks cap formation reversibly in the presence of external calcium, and then progressively lower the calcium concentration in the medium, we can determine the concentration at which the onset of inhibition of capping occurs. In these short-term experiments (6.5 min exposure to A23187) concentrations of $30\ \mu\text{M}$ -ionophore had only small effects on immunoglobulin capping when the free, extracellular $[\text{Ca}^{2+}]$ was reduced to less than $1\ \mu\text{M}$ by the addition of EGTA, but caused 80% inhibition of capping in media containing $30\ \mu\text{M}$ free Ca^{2+} (Fig. 4). This indicates that an influx of extracellular calcium rather than a release from intracellular pools is responsible for the inhibition of capping. The inhibition was fully reversible after 6.5 min exposure to A23187 by washing, but longer incubation with $30\ \mu\text{M}$ -A23187 of more than 10 min lead to partial inhibition of capping. The effect of free Ca^{2+} concentration on capping at a constant ionophore concentration of $30\ \mu\text{M}$ either in normal medium or in high- K^+ medium is shown in Fig. 4. A 50% inhibition of immunoglobulin capping was obtained at $10\text{--}20\ \mu\text{M}$ free Ca^{2+} , with significant inhibition (>30%) observed reproducibly at $10\ \mu\text{M}$ free Ca^{2+} .

Under these conditions, the free cytoplasmic calcium concentration might be affected by other cation exchanges catalysed by A23187. Data provided by Pfeiffer & Lardy (1976) indicate that the ionophore mainly induces a $\text{Ca}^{2+}/2\text{H}^+$ exchange, and since the ionophore also has a low

Fig. 4. Effect of free extracellular calcium concentration on FITC-RaMlg capping at 37°C on mouse spleen cells in the presence of 30 μM-A23187

Cells were prepared in either inorganic salts of normal RPMI 1640 (○ and △) or high (120 mM) K⁺ (● and ▲). Two methods were used to obtain the free Ca²⁺ concentrations; △ and ▲, CaCl₂ was added to the inorganic salts medium and the total Ca²⁺ measured by atomic-absorption spectroscopy; ○ and ●, the media were buffered with 10 mM-EGTA and the required CaCl₂ (calculated by an iterative computer program) was then added (see the Materials and methods section).

affinity for Na⁺ and K⁺ it could catalyse Ca²⁺/2K⁺ or Ca²⁺/2Na⁺ exchange. It is therefore conceivable that in the presence of the ionophore the existence of outwardly directed H⁺ or K⁺ gradients could lead to accumulation of a higher free cytoplasmic calcium concentration in the cell than in the external medium. The data in Fig. 4 show, however, that the effect of ionophore and extracellular Ca²⁺ on surface immunoglobulin capping is not significantly different when high K⁺ medium is substituted for normal medium. Furthermore the inhibition of capping by this method is, within experimental error (±5%), insensitive to the pH of the medium over the range 6.7–7.8 (results not shown). The lack of effect of external pH or K⁺ concentration suggests that, in the short-term at least, the H⁺ and K⁺ gradients do not significantly modify the rise in cytoplasmic Ca²⁺ concentration induced by the ionophore. It is therefore reasonable to assume that in the presence of high ionophore concentrations the free cytoplasmic calcium concentration is not very different from the concentration in the external medium. We conclude that since the entry of calcium is required to inhibit capping and we are unable to detect any inhibition of capping at the optimal concentrations of mitogens for stimulation, any increase that occurs in the free calcium concentration in the cytoplasm does not exceed 10 μM when the cells are triggered by mitogens to enter the cell cycle.

Discussion

Taken together, the data strongly imply that the cells require prolonged interaction with a well-defined concentration range of ionophore in the presence of extracellular calcium for maximal mitogenic stimulation to occur. Commitment of the maximum proportion of pig lymphocytes requires exposure to the optimal ionophore concentration for at least 20 h, with no significant stimulation detectable within 3 h of exposure. Lowering the optimal cellular A23187 concentration by washing (or by the addition of serum to cells incubated in serum-free medium) after about 24 h reduces subsequent cell death when exposure is continued throughout the incubation (48 h), and therefore increases the observed stimulation. By using this 'rescue' technique, we previously obtained small but significant stimulations by ionophore of BALB/c splenic lymphocytes, up to 15% of the level obtained with pig lymphocytes (Hesketh *et al.*, 1977). This may be significant in relation to the calcium hypothesis, in that a non-toxic calcium ionophore should be a mitogen for all types of lymphocyte. We assume that the previously reported inability of A23187 to stimulate mouse or rat lymphocytes can be attributed to its enhanced toxicity for cells of these species (Kaiser & Edelman, 1977; Hesketh, 1979).

Exposure of pig lymphocytes to at least 10-fold optimal ionophore concentrations for 2 h is not lethal to most of the cells. It is clear, however, that cells are only subsequently stimulated after washing if the residual concentration of cellular ionophore falls within the range 3–15 amol per cell. Lowering the cellular concentration of ionophore below this critical range completely prevents mitogenic action. The presence of sufficient EGTA to chelate all of the calcium in the medium during the 2 h exposure to supraoptimal ionophore before washing has no effect on the subsequent mitogenic stimulation, provided that Ca²⁺ is restored to the medium when the cells are washed at 2 h and that the residual ionophore concentration is in the appropriate range. Thus the large Ca²⁺ fluxes induced by exposure to supra-optimal ionophore concentrations for 2 h are irrelevant to the subsequent mitogenic stimulation.

Although it remains possible that the mitogenic action of the ionophore is unrelated to any effect it may have on the free cytoplasmic Ca²⁺ concentration, the requirement for prolonged exposure of the cells to the ionophore in the presence of external Ca²⁺ is consistent with the calcium hypothesis. Previous data for ⁴⁵Ca²⁺ fluxes have indicated that optimal concentrations of ionophore for mitogenic stimulation are normally just below the levels at which the ionophore has a detectable effect on the ⁴⁵Ca²⁺ fluxes in resting control cells (Hesketh *et al.*, 1977). From the present study we conclude that the large Ca²⁺ influxes that occur on short-term

exposure to supraoptimal concentrations of A23187 cannot generate a signal that commits the cells to enter the cell cycle. In terms of the calcium hypothesis, we assume that after the ionophore concentration has been reduced by repeated washing to suboptimal concentrations for stimulation, the cells recover from the large calcium fluxes by sequestering and/or pumping out the excess calcium. This eventually lowers the cytoplasmic calcium concentration back to resting (G_0) levels in the presence of residual, suboptimal, ionophore concentrations and re-addition of ionophore is required for subsequent stimulation. The experiments on the effects on capping of high ionophore concentrations in the presence of external calcium indicate that in cells that are mitogenically stimulated the free cytoplasmic Ca^{2+} concentration does not rise as high as $10\mu\text{M}$. Elsewhere we have shown that under conditions where the ionophore blocks cap formation, the drop in ATP level in the cells is a sufficient (though not necessarily unique) explanation for the inhibition of capping (Pozzan *et al.*, 1980). This inhibition of cap formation also coincides approximately with a detectable increase in $^{45}\text{Ca}^{2+}$ uptake due to the ionophore (Hesketh *et al.*, 1977), and it has been shown for isolated mitochondria from many tissues that ATP synthesis is inhibited by rapid calcium uptake with a K_m of $1\text{--}50\mu\text{M}$ (Carafoli & Crompton, 1976). Experiments on O_2 consumption of mouse splenic lymphocytes in the presence of oligomycin suggest that the drop in ATP levels in the cells in the presence of supraoptimal A23187 concentrations that inhibit capping could be due to a direct effect of calcium on mitochondrial ATP synthesis (T. Pozzan, unpublished work).

Finally we note that the use of the ionophore in these experiments to attempt to clamp the cytoplasmic calcium concentration represents a limited extension of the use of the ionophore as a chemical clamp, introduced by Lew and colleagues for the red blood cell (Ferreira & Lew, 1976). However, there are major limitations to the technique as used in the present study, compared with the precise control of cytoplasmic Ca^{2+} concentration demonstrated for the red blood cell. The present experiments have not been performed under defined steady-state conditions and therefore the equilibrium condition that

relates the free internal Ca^{2+} concentration, the concentration of external Ca^{2+} and the H^+ internal and external concentrations is not applicable to the lymphocyte cell system. The ΔpH value for the lymphocyte plasma membrane is not known, although the lack of any major effect of the external pH on the inhibition of capping suggests that, in the short-term, ΔpH may not play an important role in determining the free internal Ca^{2+} concentration. The method therefore allows only an approximate estimate of the upper limit of $10\mu\text{M}$ for the increase in free cytoplasmic Ca^{2+} concentration that might occur on mitogenic stimulation.

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