Duration of the calcium signal in the mitogenic stimulation of thymocytes

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An increase in the free cytoplasmic Ca^{2+} concentration in thymocytes can be detected by the fluorescent indicator quin 2 within a few seconds of the addition of concanavalin A and the response is quantified from the increased proportion of quin 2 in the cells chelated by Ca^{2+} ('% Ca-quin 2'). The % Ca-quin 2 in untreated cells is $53 \pm 6\%$, increases to $64 \pm 7\%$ immediately after the addition of concanavalin A and declines spontaneously over 24 h back to the level in untreated cells ($53 \pm 6\%$). The increase in % Ca-quin 2 in response to concanavalin A is completely blocked when 50 mM- α -methyl D-mannoside is added before concanavalin A and completely reversed when the competing sugar is added immediately after the mitogen. Addition of α -methyl D-mannoside at increasing intervals after concanavalin A addition causes a progressively smaller decrease in % Ca-quin 2 and has a negligible effect after 24 h, when the % Ca-quin 2 is the same as that in untreated cells. The decline in the calcium signal defined by these experiments has a similar time course to cap formation by concanavalin A on the cells. It is concluded that the calcium signal lasts only while concanavalin A is bound to the cell surface and is terminated either by capping or by the addition of α -methyl D-mannoside.

In a working hypothesis for the mitogenic stimulation of lymphocytes we have proposed that a small increase in the free cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) within the range 0.1–1 μM is an obligatory activating signal (Metcalfe *et al.*, 1980). We also proposed that the increase in $[\text{Ca}^{2+}]_i$ must be maintained for prolonged periods of up to 24 h to commit the maximum proportion of the cells to DNA synthesis, which is initiated about 24 h after the addition of mitogen.

McClain & Edelman (1976) showed that there was a requirement for prolonged interaction of the mitogen concanavalin A with human blood lymphocytes to commit most of the cells to DNA synthesis. Removal of concanavalin A from the cell surface by the addition of α -methyl D-mannoside, which competes for the concanavalin A binding sites, blocked mitogenic stimulation completely when added soon after concanavalin A. Addition of α -methyl D-mannoside at increasing intervals after concanavalin A had a progressively diminishing effect on subsequent DNA synthesis as the cells proceeded to the lectin-independent phase of mitogenesis. Subse-

quently we found that the time course of inhibition by α -methyl D-mannoside of the stimulation of mouse thymocytes by concanavalin A coincided closely with the time course of cap formation by concanavalin A on the cells (Pozzan *et al.*, 1981). By 20–24 h after the addition of concanavalin A, the maximum number of caps were formed and the addition of α -methyl D-mannoside had no effect on subsequent DNA synthesis. Since cap formation removes concanavalin A and its receptors from the cell surface, we proposed that this terminated the primary mitogenic signal, which is activated directly by the interaction of concanavalin A with the mitogenic receptors (Pozzan *et al.*, 1981).

Direct evidence that an increase in $[\text{Ca}^{2+}]_i$ is an early response to mitogens in lymphocytes has recently been obtained from the use of quin 2, a fluorescent intracellular indicator of $[\text{Ca}^{2+}]_i$ (Tsien *et al.*, 1982; Hesketh *et al.*, 1983). In thymocytes loaded with quin 2 and stimulated by concanavalin A, $[\text{Ca}^{2+}]_i$ increases by approx. 2-fold from 100 nM over the first 2 min after the addition of mitogenic concentrations of the lectin. However, the duration of this calcium signal and its relationship to the time course of cap formation have not been established. In the present paper we describe the use of quin 2 to follow the duration of the increase in $[\text{Ca}^{2+}]_i$ over the first 24 h of mitogenic stimulation by con-

Abbreviations used: $[\text{Ca}^{2+}]_i$, free cytoplasmic Ca^{2+} concentration; WGA, wheat germ agglutinin; Rhod-Con A, tetramethylrhodamine-labelled concanavalin A; FITC-WGA, fluorescein isothiocyanate-labelled wheat germ agglutinin.

canavalin A. We show that the increase in $[Ca^{2+}]_i$ immediately after the addition of mitogen can be completely reversed by the addition of α -methyl D-mannoside and that this can be used to follow the decline in the calcium signal with time. At optimal concentrations of concanavalin A for mitogenic stimulation, the rate of this decline in the calcium signal measured by using α -methyl D-mannoside is similar to the rate of cap formation by the concanavalin A, which defines the progression of the cells to the lectin-independent phase of mitogenesis. The comparison between the duration of the calcium signal and the time course of cap formation is also made for WGA. This ligand causes similar early metabolic stimulation of thymocytes to concanavalin A, but WGA is non-mitogenic and has previously been shown to cap very rapidly at concentrations that cause an increase in $[Ca^{2+}]_i$ (Hesketh *et al.*, 1983).

Materials and methods

Cell preparation

Thymocytes were prepared from 4–6 week-old BALB/c mice by teasing the thymus into a standard medium containing RPMI 1640 (Flow) supplemented with 2 mM-glutamine, 10 μ g of gentamycin/ml, 24 mM- $NaHCO_3$ and 20 mM-Tris/HCl, pH 7.2. The cells were centrifuged (500 g; 5 min) and suspended in standard medium; viability measured by Eosin exclusion was >95%. The cells were incubated throughout all experiments (except during the quin 2 fluorescence measurements in the spectrofluorimeter) in a humidified atmosphere of air/ CO_2 (19:1) at 37°C.

Quin 2 loading and fluorescence measurements

At the required times, 10 μ M- $[^3H]$ quin 2 acetoxy-methyl ester [2.42 Ci/mol; prepared as described previously (Hesketh *et al.*, 1983)] was added from a stock 10 mM solution in dimethyl sulfoxide to 6×10^6 cells in 1 ml of standard medium in glass culture tubes and incubated as above for a further 30 min before centrifugation (500 g; 5 min) and suspension in the medium for fluorescence measurements containing the inorganic salts of RPMI 1640 without Phenol Red [103 mM-NaCl, 5.6 mM- Na_2HPO_4 , 5.36 mM-KCl, 0.41 mM- $MgSO_4 \cdot 7H_2O$, 0.42 mM- $Ca(NO_3)_2 \cdot 4H_2O$] and supplemented with 11 mM-glucose and buffered with 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.3. After a further 30 min at 37°C the cells were washed as before and suspended at 6×10^6 cells/ml in the medium for fluorescence measurements. Cell suspensions were equilibrated with continuous stirring in 1 cm pathlength cuvettes for 10 min at 37°C in a Perkin-Elmer model 44B spectrofluorimeter before measurements of $[Ca^{2+}]_i$ (excitation at 339 nm; emission at 492 nm). Additions of con-

canavalin A or WGA (Miles Yeda) were made from stock solutions in standard medium (1 and 10 mg/ml respectively). Additions of 50 mM- α -methyl D-mannoside or -N-acetylglucosamine were from a stock solution of concentration 1 M in water that had been treated with Chelex 100 to remove any ions that quenched fluorescence. The % Ca-quin 2 level (the proportion of quin 2 in the cells chelated by Ca^{2+}) was calibrated by the addition of 0.05% Triton X-100 and 0.1 mM- $MnCl_2$ as described previously (Hesketh *et al.*, 1983) and $[Ca^{2+}]_i$ calculated using a value for quin 2 of $\log K_{Ca} = 7.05$.

Rate of capping

Estimation of the rate of cap formation on thymocytes was made by incubating 200 μ l samples of cells (6×10^6 cells/ml) in standard medium in flat-bottomed Microplate wells (Nunc) for various times at 37°C with Rhod-Con A [prepared by the method of Rinderknecht (1962)] or FITC-WGA (Miles Yeda). Cell samples were washed, fixed in 1% paraformaldehyde and examined on a Leitz inverted-stage fluorescence microscope. Caps, defined by fluorescent staining restricted to less than half of the cell surface, were expressed as a percentage of the total number of cells stained by the ligand (at least 200 cells were scored for each sample).

$[^3H]$ Acetylconcanavalin A binding

To estimate the concanavalin A displaced by α -methyl D-mannoside, $[^3H]$ acetylconcanavalin A (sp. radioactivity 32 Ci/mmol; Amersham International) was added to 6×10^6 cells in 1 ml of standard medium in glass culture tubes and incubated for 30 min before washing twice by centrifugation (500 g; 5 min) in 1 ml of standard medium. Cell samples (100 μ l) were treated with 50 mM- α -methyl D-mannoside and incubated for 10 min. The cells were separated from unbound $[^3H_2]$ acetylconcanavalin A by centrifugation at 15 000 g for 30 s through a barrier of silicone oil (Versilube F 50) into 20 μ l of 98% $HClO_4$ in polypropylene microcentrifuge tubes (Beckman PRO-22). After centrifugation a sample of each supernatant solution was taken before the remaining contents of the tubes were frozen by immersion in liquid N_2 . The bottom 9 mm of each tube, containing the cells dissolved in $HClO_4$, was amputated and counted for radioactivity in a Packard Tri-Carb 460C liquid-scintillation counter.

$[^3H]$ Thymidine uptake

$[^3H]$ Thymidine (1.5 μ Ci/sample; Amersham International) was added to 6×10^6 cells in 1 ml of standard medium in glass culture tubes after incubating for 43 h and at 48 h the cells were harvested by filtration through Whatman GF/C glass Micro-fibre filters and counted for radioactivity.

Results

Reversal by α -methyl D-mannoside of $[Ca^{2+}]_i$ increase by concanavalin A

When thymocytes loaded with quin 2 are stimulated by the addition of mitogenic concentrations of concanavalin A ($1\mu\text{g/ml}$), the fluorescence intensity of the intracellular quin 2 rises to a new stable level over about 2 min (Fig. 1a). The proportion of Ca-quin 2 rises from $53 \pm 6\%$ ($n = 20$) to $64 \pm 7\%$, corresponding to an increase in $[Ca^{2+}]_i$ from about 100 to 200 nM. The subsequent addition of 50 mM- α -methyl D-mannoside produces an immediate decrease in fluorescence, which returns to the initial level before concanavalin A addition ($53 \pm 6\%$ Ca-quin 2) over about 5 min. The addition of α -methyl D-mannoside to cells without concanavalin A produces no change in $[Ca^{2+}]_i$ and completely blocks the increase in $[Ca^{2+}]_i$ when concanavalin A is added subsequently (Fig. 1b). These effects of α -methyl D-mannoside are attributable to the competition for the concanavalin A binding sites, so that the binding of concanavalin A to glycoproteins on the cell surface is reversed or blocked and $[Ca^{2+}]_i$ is held at the resting level. The competitive displacement of concanavalin A from the cells by α -methyl D-mannoside can be shown by the use of $[^3\text{H}]$ acetylconcanavalin A, which has the same mitogenic dose-response profile as concanavalin A and induces the same changes in $[Ca^{2+}]_i$ in

thymocytes. The addition of 50 mM- α -methyl D-mannoside to cells washed 30 min after the addition of $1\mu\text{g}$ of $[^3\text{H}]$ acetylconcanavalin A/ml results in the immediate displacement of at least 70% of the bound $[^3\text{H}]$ acetylconcanavalin A. The subsequent mitogenic stimulation of the cells measured as $[^3\text{H}]$ thymidine incorporation between 42 and 48 h is inhibited by $>95\%$, consistent with data from previous studies (Pozzan *et al.*, 1981).

Duration of $[Ca^{2+}]_i$ increase after the addition of concanavalin A and WGA

Direct measurements of $[Ca^{2+}]_i$ in thymocytes from the % Ca-quin 2 can give variations between separate experiments that are comparable with the changes in % Ca-quin 2 after the addition of concanavalin A shown in Fig. 1(a). Thus absolute measurements of $[Ca^{2+}]_i$ made by measuring the % Ca-quin 2 in cells that were loaded with quin 2 at increasing intervals after the addition of concanavalin A are not an accurate method of following the change in $[Ca^{2+}]_i$ as a function of time. However, large numbers of determinations have defined the % Ca-quin 2 in unstimulated cells as $53 \pm 6\%$ ($n = 20$), at equilibrium after the addition of concanavalin A as $64 \pm 7\%$ ($n = 20$) and at 24 h after the addition of concanavalin A as $55 \pm 3\%$ ($n = 4$). These correspond to $[Ca^{2+}]_i$ values of 124 nM, 196 nM and 134 nM respectively. The data therefore show that 24 h after concanavalin A addition the indicated

Fig. 1. The effect of concanavalin A and α -methyl D-mannoside on the fluorescence of mouse thymocytes loaded with $1\text{ mM-}[^3\text{H}]$ quin 2

(a) Addition of optimally mitogenic concanavalin A ($1\mu\text{g/ml}$; Con A) followed by 50 mM- α -methyl D-mannoside (α -MeMann); (b) addition of 50 mM- α -methyl D-mannoside followed by $1\mu\text{g}$ of concanavalin A/ml. The % Ca-quin 2 is calculated as $[(I - 0.16\Delta I)/0.84\Delta I] \times 100$ after the addition of 0.05% Triton X-100 and 0.1 mM- Mn^{2+} as shown in (b); I and ΔI are the fluorescence intensities illustrated and $0.16\Delta I$ is the fluorescence intensity of the free quin 2 tetravalent anion or the Mg^{2+} -quin 2 complex at pH 7.1.

$[Ca^{2+}]_i$ has returned to the level before concanavalin A addition. Furthermore linear regression analysis of data from 18 determinations at intermediate times shows a progressive decline in $[Ca^{2+}]_i$ over the initial 24 h after the addition of mitogen. However, measurements of the decrease in % Ca-quin 2 after addition of α -methyl D-mannoside to cells loaded with quin 2 at increasing intervals after the addition of concanavalin A provide a more accurate measure of the concanavalin A-induced calcium signal, because relative measurements of the change in % Ca-quin 2 are made for each cell sample. Data from these experiments in Fig. 2 show that the decrease in % Ca-quin 2 after addition of 50 mM- α -methyl D-mannoside becomes progressively smaller over a period of 24 h.

We conclude that 24 h after the addition of concanavalin A $[Ca^{2+}]_i$ has returned to the level in untreated cells and is then unaffected by the addition of α -methyl D-mannoside. The lack of response at this time is not due to insufficient concanavalin A in the medium: throughout the 24 h after the addition of 1 μ g of concanavalin A/ml, addition of a further 10 μ g/ml has no effect on the % Ca-quin 2. Thus all of the concanavalin A receptors that mediate the increase in $[Ca^{2+}]_i$ in the cells remain saturated throughout the 24 h.

Similar data for the duration of the calcium signal caused by WGA are also shown in Fig. 2. Concentrations of 10 μ g of WGA/ml produce a maximal increase in $[Ca^{2+}]_i$ comparable with that of concanavalin A (Hesketh *et al.*, 1983). However, the decrease in % Ca-quin 2 caused by the addition of 50 mM-*N*-acetylglucosamine after WGA declines very rapidly to a negligible effect over about 2 h, and

Fig. 2. The decrease in % Ca-quin 2 in thymocytes due to the addition of 50 mM- α -methyl D-mannoside (●) or 50 mM-*N*-acetylglucosamine (○) to cells treated with 1 μ g of concanavalin A/ml or 10 μ g of WGA/ml respectively at time zero

The regression lines were obtained by the least-squares method and the correlation coefficients are 0.89 (●) and 0.59 (○).

the % Ca-quin 2 returns to the resting level ($53 \pm 6\%$) over the same period.

Time course of cap formation by concanavalin A and WGA

We have shown previously that the time course of commitment of thymocytes to DNA synthesis by concanavalin A coincides with the time course of cap formation by concanavalin A on the cell surface (Pozzan *et al.*, 1981). Data for these responses were obtained under experimental conditions directly comparable with those for the % Ca-quin 2 data in Fig. 2. The time course of commitment to DNA synthesis was determined by the addition of 50 mM- α -methyl D-mannoside at increasing times up to 24 h after the addition of Rhod-Con A (Fig. 3a). This

Fig. 3. Commitment to DNA synthesis and rate of cap formation by ligands on thymocytes

(a) shows the time course of commitment to DNA synthesis in thymocytes measured by $[^3H]$ -thymidine uptake between 42 and 48 h. Optimally mitogenic Rhod-Con A (1 μ g/ml) was added at time zero to 6×10^6 cells/ml followed by 50 mM- α -methyl D-mannoside at the times indicated and stimulation is expressed as a percentage of the maximum value obtained (37×10^3 c.p.m./ 10^6 cells). (b) shows the rate of cap formation by (●) Rhod-Con A (1 μ g/ml) and (○) FITC-WGA (10 μ g/ml) on thymocytes.

may be compared with the time course of cap formation by Rhod-Con A in Fig. 3(b). The accuracy of the data for the calcium signal in Fig. 2 does not allow us to determine whether cap formation and the decrease in the calcium signal are precisely coincident. It is clear, however, that the calcium signal due to concanavalin A does not persist when maximal cap formation has occurred.

Further consistent evidence is provided by the data for cap formation by WGA, which is complete within about 2 h, coinciding with the duration of the calcium signal induced by this ligand (compare Figs. 3b and 2). We therefore attribute the rapid decline in the calcium signal to the removal of WGA and its receptors for the cell surface by cap formation.

Discussion

Taken together the data strongly suggest that the increase in $[Ca^{2+}]_i$ immediately after the addition of concanavalin A to quin 2-loaded cells decreases over 24 h back to the level in untreated cells. The time course of the decline in the calcium signal, defined by the effect of α -methyl D-mannoside, is similar to that of cap formation on the cells by concanavalin A. We therefore assume that the increase in $[Ca^{2+}]_i$ in a cell is only maintained while concanavalin A interacts directly with the cell surface (i.e., before displacement of concanavalin A by α -methyl D-mannoside or by cap formation).

The results imply that the increase in $[Ca^{2+}]_i$ is a primary response to the mitogen in that it is directly dependent on the interaction of the ligand with its receptors. This distinguishes the calcium signal from some other early metabolic responses that appear to depend directly on the interaction of concanavalin A with the receptors in the pre-capping period, but remain activated and independent of the ligand after cap formation. For example, the stimulation of glycolysis and $[^3H]$ uridine uptake are independent of concanavalin A when capping is complete after 20–24 h, but are completely dependent on concanavalin A before significant cap formation occurs (Northoff *et al.*, 1978; A. N. Corps, unpublished work). Thus we can distinguish signals, such as the increase in $[Ca^{2+}]_i$, which are switched off when commitment has occurred from those responses that eventually become independent of the ligand–receptor

interaction, and define the cells as committed to DNA synthesis. This does not of course preclude the expression of new receptors at the cell surface, which are necessary to maintain the commitment of the cells after they become independent of concanavalin A (e.g., receptors for T cell growth factor, which appear in G_1). We note that T lymphocytes maintained for prolonged periods in culture become independent of the primary mitogen, but are absolutely dependent on the presence of endogenous T cell growth factor to remain in the cell cycle (Larsson *et al.*, 1980). The present data suggest that these secondary mitogens do not cause an increase in $[Ca^{2+}]_i$ since the $[Ca^{2+}]_i$ appears to return to the normal level in resting cells once the cells become independent of the primary mitogen. This is consistent with the limited evidence from *Xenopus* oocytes that there are no substantial changes in $[Ca^{2+}]_i$ during the cycle of activated cells.

If an increase in $[Ca^{2+}]_i$ in response to the primary mitogen is obligatory for subsequent commitment, there must be at least one biochemical response in the pre-capping (concanavalin A-dependent) period that is absolutely dependent on the increase in $[Ca^{2+}]_i$. An analysis of the dependence of early metabolic responses and of RNA and protein synthesis on $[Ca^{2+}]_i$ should provide a further test of the working hypothesis summarized earlier.

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