The effect of mitogenic lectins and monoclonal antibodies on intracellular free calcium concentration in human T-lymphocytes

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The effect of mitogenic lectins and the mitogenic antibody UCHT1 on the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in human lymphocytes was investigated by using the fluorescent Ca^{2+} -indicator quin2. Phytohaemagglutinin, concanavalin A and UCHT1 increased $[\text{Ca}^{2+}]_i$ in T-cells to a maximum level within 2 min. No T-cell response was seen with poke weed mitogen. None of the mitogens affected non-T-cell $[\text{Ca}^{2+}]_i$.

There is considerable evidence that an increase in the concentration of intracellular free Ca^{2+} plays an important role in the stimulation of lymphocytes (see review by Lichtman *et al.*, 1983). The relationship between such early biochemical events and the ultimate proliferative response of the cells is unclear, since it appears to involve interactions between different types of lymphocyte and accessory cells (Arala-Chaves *et al.*, 1978). A novel method for estimating $[\text{Ca}^{2+}]_i$ has been developed by Tsien *et al.* (1982a,b) based on the fluorescence of the Ca^{2+} -indicator quin2. This tetracarboxylate anion binds Ca^{2+} ions with a 1:1 stoichiometry and a concomitant 5-fold increase in fluorescence emission. Cells are loaded with quin2 by incubating them with its acetoxymethyl ester. This is able to diffuse freely across the plasma membrane and enter the cytoplasm. Here the ester is hydrolysed by endogenous esterases to produce the free acid which then binds Ca^{2+} .

Tsien *et al.* (1982a) have investigated the effect of the mitogens concanavalin A and PHA on murine and porcine lymphocytes and have shown that a 2-fold increase in average $[\text{Ca}^{2+}]_i$ is induced within 2–3 min. It was inferred from these data that the elevation of $[\text{Ca}^{2+}]_i$ in the responding population was much higher, since only T-cells are expected to react to these stimuli. We have studied the effect of mitogens on lymphocytes from human peripheral blood by using quin2 and show that PHA, concanavalin A and a mitogenic monoclonal antibody,

UCHT1, induce an increase in $[\text{Ca}^{2+}]_i$ within 2 min of treatment. Using fractionated cell populations, we also show that only T-cells increase $[\text{Ca}^{2+}]_i$ in response to these mitogens. Our data support the view that an increase in $[\text{Ca}^{2+}]_i$ is a primary event in the initiation of human T-cell activation.

Experimental

Cell preparation

Mononuclear cells from peripheral blood taken from healthy volunteers were separated by buoyant density sedimentation (Boyum, 1968) using Ficoll-Hypaque (LSM, Flow Laboratories). After washing in RPMI 1640H medium (Gibco), monocytes were removed by adherence to plastic Petri dishes in an overnight incubation. T-cells and non-T-cells were separated by rosetting, using AET (2-aminoethylisothiuronium bromide hydrobromide)-treated sheep erythrocytes (Kaplan & Clark, 1974) followed by centrifugation over Ficoll-Hypaque. Cells collecting at the interface (non-T-cells, E^- fraction) were removed and washed twice in RPMI medium. T-cells (E^+ fraction) were recovered from the pelleted rosettes by lysis of the red cells with NH_4Cl (Lysing Reagent, Ortho Diagnostics) and then washed three times. The remaining cells (E^+ fraction) were washed three times. Both the E^+ and E^- samples were resuspended in RPMI medium supplemented with 1% (w/v) bovine serum albumin (RIA grade, Sigma). T-cells were also prepared by elimination of the non-T subpopulations by using an immune rosetting technique. Mononuclear cells were treated with a mixture of the following mouse monoclonal

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free $[\text{Ca}^{2+}]$; PHA, phytohaemagglutinin; PWM, poke weed mitogen; DTPA, diethylenetriaminepenta-acetic acid.

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Table 1. *Monoclonal antibodies*

Name	Specificity	Source
BA1	B-cells, granulocytes	J. H. Kersey*
B1	B-cells	L. M. Nadler†
UCHM1	Monocytes	P. C. L. Beverley‡
UCHT1	T3 antigen, (pan-T)	P. C. L. Beverley§
UCHT2	T1 antigen, (pan-T)	P. C. L. Beverley§
Leu 5	Sheep erythrocyte receptor (pan-T)	Becton Dickinson
Leu 2a	T-cells (cytotoxic/suppressor subset)	Becton Dickinson
OKT4	T-cells (helper subset)	Ortho Diagnostics
OKM3	Monocytes	Ortho Diagnostics
H366	NK-cells, 50% of monocytes, 10% E ⁺ cells (mainly UCHT1 ⁺)	P. C. L. Beverley
2A1	HLA-A, -B, -C (non-polymorphic region)	P. C. L. Beverley§

* Abramson *et al.* (1981).† Nadler *et al.* (1981).‡ Mitchell *et al.* (1983).

§ Beverley (1982).

|| Bai *et al.* (1983).

antibodies: BA1, B1, OKM3, UCHM1 and H366. These are described in Table 1. The antibody-coated cells were then mixed with ox red blood cells to which antimouse immunoglobulin had been coupled (Indiveri *et al.*, 1979). Rosettes were removed by centrifugation on iso-osmotic Percoll (sp. gr. 1.085, Pharmacia) and the T-cells (I⁻ cells) were collected from the interface.

Quin2 loading and fluorescence measurements

Quin2 acetoxymethyl ester (Lancaster Synthetics) dissolved in dimethyl sulphoxide was added to cells in the RPMI/albumin medium (10⁷ cells/ml) to give a final concentration of 25 µM. This suspension was incubated at 37°C for 20 min and then diluted 5-fold with RPMI/albumin medium before a further 40 min of incubation. Finally the cells were spun down (350g for 10 min at 18°C) and resuspended in RPMI/albumin at (2–5) × 10⁶ cells/ml.

Immediately before fluorescence measurement an aliquot of this suspension was centrifuged [(2–3) × 10⁶ cells, 200g for 10 min at room temperature] and the cells were resuspended in 1 ml of a buffered salt solution containing 145 mM-NaCl, 5 mM-KCl, 0.5 mM-Na₂HPO₄, 1 mM-CaCl₂, 5 mM-glucose and 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] titrated to pH 7.4 at 37°C with NaOH. This suspension was preincubated in quartz tubes at 37°C for 10–15 min before measurement. Fluorescence was measured using an Aminco-Bowman spectrofluorimeter (SPF), with the sample compartment at 37°C. Excitation was set to 339 nm (2 mm slits) and emission to 500 nm (4 mm slits). An additional 418 nm long-pass filter (KV418, Schott) was placed in front of the photomultiplier tube to reduce light scatter arte-

facts. Under continuous illumination conditions without stirring, the fluorescence signal from quin2-loaded lymphocytes faded. If the shutter in the excitation path was closed and reopened after a few minutes, no change in the emission intensity was detected, indicating that the decay was due to photobleaching of dye in the cells occupying the light beam. This fading was minimized by occasional stirring. Addition of 0.5 mM-MnCl₂ to loaded cells produced no drop in emission, indicating that the amount of extracellular quin2 was negligible. The percentage saturation of quin2 by Ca²⁺ in the cells was estimated by the method of Hesketh *et al.* (1983). The Ca²⁺-saturated quin2 fluorescence level was calibrated by addition of 0.05% (v/v) Triton X-100 (Sigma) and 10 µM-DTPA (Sigma) to permeabilize the cells and scavenge heavy metal ions respectively. The background signal due to autofluorescence and light scatter was determined by adding 0.5 mM-MnCl₂. [Ca²⁺]_i was calculated assuming a Ca²⁺-quin2 dissociation constant of 115 nM (Tsien *et al.*, 1982b).

Immunofluorescence analysis

The cell preparations obtained by rosetting were analysed by flow cytometry (FACS IV, Becton-Dickinson) using indirect immunofluorescence as previously described (Linch *et al.*, 1982). An average of 70% of unfractionated cells reacted with UCHT1. Analysis of the E⁺ fractions showed that over 90% of the cells were positive with UCHT1. By contrast, the E⁻ fraction contained an average of 5% UCHT1⁺ cells. The concentration of each of the monoclonal antibodies required to saturate the cell-surface binding sites was also determined by flow cytometric analysis of indirect immunofluorescence.

Cell proliferation assay

Optimally mitogenic concentrations of concanavalin A, PHA, PWM and UCHT1 were determined by measuring the incorporation of ^{125}I -labelled deoxyuridine (Elves, 1969).

Lectins

Concanavalin A and PWM were obtained from Sigma. PHA (reagent grade) was obtained from Wellcome Laboratories. The optimum dose was found to be $20\text{ }\mu\text{g/ml}$ for concanavalin A, 5 mg/ml for PWM and $10\text{ }\mu\text{l/ml}$ for PHA.

Results

Lymphocyte $[\text{Ca}^{2+}]_i$

The resting $[\text{Ca}^{2+}]_i$ in mononuclear cells from seven individuals was measured in 56 experiments on 16 different occasions. A value of $107 \pm 6\text{ nM}$ (S.E.M., $n = 16$) was obtained. Removal of adherent cells (nine experiments) did not affect this value. On eight occasions the resting $[\text{Ca}^{2+}]_i$ in unfractionated lymphocytes was compared with that of E^+ cells from the same individual. In these experiments the $[\text{Ca}^{2+}]_i$ in the E^+ cells was $67 \pm 6\text{ nM}$ compared with $96 \pm 7\text{ nM}$ in the unfractionated cells. The $[\text{Ca}^{2+}]_i$ in the non-T-cell fraction was $150 \pm 39\text{ nM}$ ($n = 5$). To determine whether the lower value in the E^+ cells was due to an intrinsic property of T-cells or to the fractionation procedure, T-cells were also prepared by an immune rosetting technique in which the non-T-populations were removed to yield T-cells that had not been subjected to rosetting or a lysis procedure. In three experiments the unfractionated cells had a mean $[\text{Ca}^{2+}]_i$ of $103 \pm 31\text{ nM}$ and the I^- cells gave a value of $101 \pm 40\text{ nM}$, suggesting that the lower $[\text{Ca}^{2+}]_i$ found in the E^+ cell fraction was at least in part due to the fractionation procedure.

Effect of plant lectins

Optimal and twice-optimal mitogenic concentrations of concanavalin A and PHA produced an increase in the fluorescence of quin2-loaded mononuclear cells. This reached a maximum in approx. 2 min and then decreased back towards the original level. Fig. 1 shows data from E^+ and E^- cells. Concanavalin A and PHA induced significantly larger increases in $[\text{Ca}^{2+}]_i$ in the E^+ fraction compared with unfractionated cells (Table 2). As well as exhibiting a lower resting level of intracellular free Ca^{2+} , the E^+ cells attained a higher $[\text{Ca}^{2+}]_i$ than unfractionated cells after stimulation. On the other hand, PWM at optimal and twice-optimal mitogenic concentrations produced no change in $[\text{Ca}^{2+}]_i$ in either the T-cell or non-T-cell fraction during the 10 min observation period. Subsequent challenge with UCHT1 gave rise to the expected

Fig. 1. Effect of mitogenic lectins on the fluorescence of quin2-loaded human lymphocytes

Fluorescence traces obtained from T-cells (E^+ cells) and non-T-lymphocytes (E^- cells) loaded with the Ca^{2+} indicator, quin2 (see the text), and treated with mitogenic doses of PHA, concanavalin A and PWM are shown. Fluorescence levels of Ca^{2+} -saturated dye (max.) were determined by addition of Triton X-100 (0.05%) and DTPA ($10\text{ }\mu\text{M}$). The background signals (min.) were determined by addition of MnCl_2 (0.5 mM). The effect of UCHT1 on E^+ cells previously treated with PWM is also shown. The lowest trace shows the absence of any effect of concanavalin A on E^- cells. Similar traces were obtained with PHA and PWM.

increase in $[\text{Ca}^{2+}]_i$ in T-cell preparations (Fig. 1). The dose of PWM used in these studies had a stimulation index of 99 after 3 days in culture.

Fig. 2. Effect of UCHT1 on quin2-loaded human lymphocytes

Fluorescence traces obtained from quin2-loaded T- and non-T-lymphocytes treated with UCHT1 are shown. Fluorescence levels of Ca^{2+} -saturated dye (max.) and background signals (min.) are indicated. The upper trace shows the effects of different concentrations of the antibody on T-cells (E^+ cells) and the lower trace shows the absence of any effect of a mitogenic dose of the antibody on non-T-lymphocytes (E^- cells).

Effect of monoclonal antibodies

The response of quin2-loaded, unfractionated cells to a saturating dose of the mitogenic antibody UCHT1 is illustrated in Fig. 2. There is an initial latent period of approx. 1 min during which there is no fluorescence change. This is followed by a moderately rapid increase in emission, reaching a maximum at about 2 min. Consistently larger responses were obtained from E^+ cell preparations but the timing of the effects was similar. UCHT1 did not elicit a response in E^- cells (Table 2).

To determine the dose-dependence of the fluorescence increase, unfractionated cells were treated with different dilutions of antibody. The UCHT1 antibody preparation used was found to give maximal stimulation at a protein concentration of $3 \mu\text{g/ml}$ (Fig. 2). An intermediate response was observed at $2 \mu\text{g/ml}$ and there was no effect at $1 \mu\text{g/ml}$. The saturating concentration of this antibody, determined by immunofluorescence analysis, was $2 \mu\text{g}$ of protein/ml, which compares well with the concentration required to produce the maximal change in $[\text{Ca}^{2+}]_i$.

The effects of saturating doses of several non-mitogenic monoclonal antibodies were also investigated. These were directed against antigens which are abundant on the plasma membrane of T-cells (see Table 1). 2A1 had no effect on the fluorescence of quin2-loaded mononuclear cells, although there are considerably more sites for this antibody on the T-cell surface than there are for UCHT1. OKT4 and Leu 2a were used simultaneously to stain all T-cells but neither had any effect on quin2 fluorescence. Leu 5 also had no effect on the emission of quin2-loaded cells. Sheep red cell fragments do not obscure the antibody binding site on E^+ cells because these preparations stained normally with Leu 5. In all these experiments subsequent treatment with UCHT1 elicited the expected increase in quin2 fluorescence.

Discussion

The value we have obtained for human lymphocyte $[\text{Ca}^{2+}]_i$ is similar to that estimated for animal lymphocytes (Tsien *et al.*, 1982b; Hesketh *et al.*, 1983). T-cells prepared by E-rosetting had a lower $[\text{Ca}^{2+}]_i$, but this was not found on T-cells prepared by negative selection using immune rosetting. This suggests that the low value in the E^+ cells may have been caused by either the rosetting procedure, passage through Ficoll-Hypaque or the hypo-osmotic lysis step. It must be noted, however, that E^- cells that had not been subjected to these

Table 2. Effect of mitogenic lectins and the antibody UCHT1 on $[\text{Ca}^{2+}]_i$ of human lymphocytes
Values are the ratios of $[\text{Ca}^{2+}]_i$ after treatment to that of the same cells before treatment, and are means $\pm$ S.E.M. for the number of observations given in parentheses.

	Unfractionated cells	E^+ cells	E^- cells
Concanavalin A	1.62 ± 0.06 (n = 5)	1.94 ± 0.21 (n = 10)	Unchanged (n = 6)
PHA	2.06 ± 0.2 (n = 15)	4.4 ± 1.1 (n = 3)	Unchanged (n = 2)
PWM	Unchanged (n = 4)	Unchanged (n = 3)	Unchanged (n = 2)
UCHT1	1.56 ± 0.04 (n = 26)	2.17 ± 0.13 (n = 22)	Unchanged (n = 5)

procedures had a higher $[Ca^{2+}]_i$ than did the unfractionated cells. This illustrates a difficulty in estimating the $[Ca^{2+}]_i$ of purified cell populations. The T-cells produced by E-rosetting are, however, fully functional and clearly respond to concanavalin A and PHA. These lectins bind to both T- and B-lymphocytes, but only T-cells are thought to respond by an early increase in Ca^{2+} uptake (Freedman *et al.*, 1975). It has been shown, using quin2, that concanavalin A and PHA induce an increase in $[Ca^{2+}]_i$ within 2–3 min in unfractionated mouse and pig lymphocytes, the average $[Ca^{2+}]_i$ rising from approx. 123 nM to between 200 and 300 nM (Tsien *et al.*, 1982a). This is similar to the rise that we have observed in human lymphocytes (Table 2). Our experiments with T-cell-enriched and depleted preparations show, moreover, that the response is specific for T-lymphocytes. The ratio of $[Ca^{2+}]_i$ after stimulation compared with the resting value is significantly greater for E^+ cells than for the unfractionated cells. Conversely, the T-cell-depleted fractions (E^-) gave no measurable fluorescence increase with these mitogens.

It is of considerable interest that in the experiments with PWM no change in $[Ca^{2+}]_i$ was detectable during the 10 min observation period. This indicates that the mechanism of action of this mitogen is distinct from that of PHA and concanavalin A. It is possible that PWM activation of human T-cells is Ca^{2+} -independent, but this is unlikely since the lectin induces a Ca^{2+} flux in murine lymphocytes (Freedman *et al.*, 1975). It is possible that the timing of the Ca^{2+} movement is different, that this discrepancy reflects a species difference, or that accessory cells are required in the early stages of activation by PWM. Cell interactions in suspension are minimal at the cell concentrations used in this study, and the rapid responses seen with PHA and concanavalin A suggest that such interactions are not involved in the early Ca^{2+} changes induced by these mitogens.

PHA and concanavalin A bind to a variety of cell surface glycoproteins present on all mononuclear cells. UCHT1, on the other hand, is a mitogenic monoclonal antibody that binds to a single, well-defined cell-surface glycoprotein, the T3 antigen (Beverley & Callard, 1981). Anti-T3 antibodies have been shown to block a variety of T-cell functions including the proliferative response to soluble antigens and the generation of cytotoxic T-cells in mixed lymphocyte culture (Reinherz *et al.*, 1980). Since these antibodies are also potent T-cell mitogens (van Wauwe *et al.*, 1980), it is very likely that the T3 molecule is involved in T-cell recognition or triggering of T-lymphocytes by antigen. Furthermore, when interleukin-2-dependent T-cell clones are exposed to clone-specific monoclonal antibody both T3 and the putative T-

cell receptor are lost together from the cell surface (Zanders *et al.*, 1983). These modulated cells still respond to interleukin-2, suggesting that T3 has a role in the initiation of activation but is not involved in maintaining clonal expansion. The biochemical mechanism by which T3 is involved in triggering remains unclear.

We show that UCHT1, at doses sufficient to saturate cell-surface binding, induces an increase in $[Ca^{2+}]_i$ in T-cells but not in non-T populations (E^-). A variety of monoclonal antibodies that bind to cell surface molecules other than T3 have no effect on $[Ca^{2+}]_i$. It has been shown that mitogenic stimulation of T-lymphocytes by UCHT1 requires the presence of accessory cells of monocyte-macrophage lineage (Tax *et al.*, 1983) but the speed of the response in suspensions of purified T-cells (E^+) suggests that accessory cells are also not required for the early Ca^{2+} response induced by UCHT1. This indicates that a rise in $[Ca^{2+}]_i$ is not, in itself, a sufficient signal for cell proliferation.

The ability to detect a rapid activation signal in human T-cells with quin2, combined with the development of monoclonal antibodies to T-cell antigen receptor proteins and antigen-specific T-cell clones, will be powerful tools in the dissecting of the biochemical mechanisms involved in T-cell triggering.

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