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Cyclosporine does not inhibit mitogen-induced inositol phospholipid degradation in mouse lymphocytes

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Summary. The degradation of phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol and inositol trisphosphate is elicited by ligand-receptor interactions in many cell types, and may be involved in the induction of cell growth. The mitogens concanavalin A and anti-immunoglobulin antibodies have previously been shown to induce degradation of PIP_2 in mouse thymocytes and B cells, respectively. We have now investigated the effects of the immunosuppressive peptide cyclosporine (CS) on this response, since CS appears to inhibit an early step in lymphocyte activation by mitogens that induce PIP_2 degradation and Ca^{2+} mobilization. We found that CS, at doses that completely abrogated the proliferative responses, did not affect the degradation of inositol phospholipids in either thymocytes or B cells. We therefore conclude that if PIP_2 degradation is implicated in lymphocyte activation, then CS does not interfere with the second messenger production initiated by PIP_2 breakdown, but rather with a later event(s) elicited by this pathway.

INTRODUCTION

Cyclosporine (CS), a cyclic undecapeptide of fungal origin, is a potent immunosuppressive agent which is widely used to prevent rejection of organ transplants.

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The drug inhibits activation of T-helper cells in a variety of species (Klaus, 1981; Morris, 1981), and in both mouse and man it also inhibits the activation of B cells by anti-immunoglobulin antibodies (anti-Ig) (Dongworth & Klaus, 1982; Muraguchi *et al.*, 1983). The mode of action of CS is unknown, but in several *in vitro* studies it was found that the drug blocks an early step in the activation of both T and B cells (Kronke *et al.*, 1984; Klaus & Hawrylowicz, 1984).

An early event associated with the receptor-mediated stimulation of many cell types is the degradation of plasma membrane phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate and diacylglycerol (reviewed by Berridge, 1984a). The enhanced breakdown of PIP_2 is generally accompanied by an increase in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). A substantial body of evidence now indicates that degradation of phosphoinositides (inositol phospholipids) and Ca^{2+} mobilization are probably fundamental mechanisms for the transduction of a wide variety of extracellular signals into cellular responses, including the induction of cell growth (Berridge, 1984b). In line with the latter, mitogen-induced increases in $[Ca^{2+}]_i$ and PIP_2 degradation have been demonstrated in both T and B cells (Bijsterbosch *et al.*, 1985; Taylor *et al.*, 1984; Hasegawa-Sasaki & Sasaki, 1983; Tsien, Pozzan & Rink, 1982; Imboden & Stobo, 1985).

The likely importance of phosphoinositide degradation and Ca^{2+} mobilization in the induction of lymphocyte growth, together with the lipophilic

nature of CS, suggested the attractive possibility that this drug interferes with one (or both) of these events. This hypothesis is supported by recent data that indicate that susceptibility to inhibition by CS is restricted to those lymphocyte activators that induce phosphoinositide degradation and/or Ca^{2+} mobilization (Bijsterbosch *et al.*, 1985; Kay, Meehan & Benzie, 1983b; Kay, Benzie & Borghetti, 1983a). We have therefore studied the effects of CS on PIP_2 degradation in mouse thymocytes and B cells.

MATERIALS AND METHODS

Animals

In all experiments, male (CBA × C57BL/6)F_1 mice, bred under specific pathogen-free conditions at the National Institute for Medical Research, were used.

Reagents

[2-^{3}H]inositol (15 Ci/mmol) and [6-^{3}H]thymidine (5 Ci/mmol) were obtained from Amersham International, Amersham, Bucks. Concanavalin A was from Sigma, St Louis, MO. Affinity-purified F(ab')$_2$ fragments of rabbit anti-mouse Fab were prepared as described previously (Hawrylowicz, Keeler & Klaus, 1984). Cyclosporine (kindly provided by Dr J. F. Borel, Sandoz Ltd, Basel, Switzerland) was dissolved in Tween 80/ethanol (1:4, v/v) to 10 mg/ml and then further diluted in medium (Dongworth & Klaus, 1982). All other chemicals were analytical grade.

Cell preparations

B lymphocytes were prepared from spleens of 3–6-month-old mice by killing T cells and removing adherent cells as described previously (Bijsterbosch *et al.*, 1985). The resulting cell preparations contained >90% surface Ig-positive cells by immunofluorescent analysis.

Suspensions of thymocytes were prepared from 4–6-week-old mice by pressing thymuses through a stainless steel grid. The cells were layered onto discontinuous (50–85%) gradients of Percoll and centrifuged for 15 min at 1200 ***g***. Cells at the 50–85% interphase were collected. The preparations were essentially free of erythrocytes and dead cells, and contained >95% Thy 1-positive cells by immunofluorescent analysis.

Assay of DNA synthesis

Aliquots of 10^6 B cells or thymocytes were cultured in flat-bottomed microtitre wells in 0·2 ml RPMI-1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 1 mM pyruvate, non-essential amino acids, penicillin, streptomycin and 5% (v/v) fetal calf serum (complete medium). Cultures were maintained at 37° in a humidified atmosphere containing 6·5% CO_2. After 68 hr of incubation, 0·5 μCi of [^{3}H]thymidine was added, and incorporation of label into DNA was determined 4 hr later by harvesting on glass fibre discs followed by liquid scintillation counting.

Assay of inositol phosphates

B cells and thymocytes, suspended to 8×10^7/ml in Hanks' balanced salt solution containing 0·5% (w/v) gelatin and buffered to pH 7·2 with 20 mM HEPES, were labelled with [^{3}H]inositol (0·5 μCi/10^6 cells) for 4 hr at 37° in a 6·5% CO_2 atmosphere. After labelling, the cells (>95% viable) were washed and resuspended to $5{\cdot}6 \times 10^6$ per ml in complete medium. Aliquots of 0·54 ml were preincubated for 30 min (B cells) or 2 hr (thymocytes) at 37° in a 6·5% CO_2 atmosphere. Then, the cells were stimulated by adding either anti-Ig or Con A, in 60 μl of medium. The incubations were terminated by adding successively 0·12 ml of 0·22 N HCl and 2·7 ml of chloroform/methanol (1:2; v/v). Phases were separated by adding 0·9 ml of chloroform and 0·9 ml of water. Aliquots of 2–3 ml of the upper phase were diluted eight-fold with water and applied to columns containing approximately 0·5 ml of Dowex-1 in the formate form. [^{3}H]inositol and [^{3}H]glycerophosphoinositol were eluted with 10 ml volumes of water and 5 mM disodium tetraborate + 30 mM sodium formate, respectively. Then, the total [^{3}H]inositol fraction (consisting of inositol -mono, -bis and -trisphosphate) was eluted with 10 ml of 0·1 M formic acid + 1·0 M ammonium formate. Validation of the method is given by Bijsterbosch *et al.* (1985).

RESULTS

Inositol phospholipid degradation in thymocytes and B cells

Induction of PIP_2 degradation by Con A in thymocytes and by anti-Ig in B cells has been demonstrated by measuring the liberation of [^{3}H]inositol (poly)phosphates from prelabelled inositol phospholipids (Bijsterbosch *et al.*, 1985; Taylor *et al.*, 1984). However, the inositol phosphates formed are rapidly dephosphorylated to free inositol, which makes the assay

relatively insensitive. Li^+ is known to inhibit inositol 1-phosphatase, which catalyses the final dephosphorylation step (Hallcher & Sherman, 1980). We have shown previously that inclusion of 5mM LiCl in the culture medium results in accumulation of inositol phosphates in B cells stimulated with anti-Ig (Bijsterbosch *et al.*, 1985). Similar results were obtained in thymocytes stimulated with Con A. In all following experiments, inositol phosphate formation was therefore assayed in the presence of 5 mM LiCl.

In B lymphocytes, the induction of inositol phosphate formation reaches a plateau at mitogenic concentrations of anti-Ig (10–50 μg/ml; see Bijsterbosch *et al.*, 1985). Con A is optimally mitogenic for thymocytes at 2 μg/ml (Fig. 1). Although inositol phosphate formation was readily detectable at this concentration, the response was only some 40% of the maximum reached at about 5 μg Con A/ml. A similar discrepancy between the dose-response curves of mitogenesis and Ca^{2+} mobilization in Con A-stimulated thymocytes was found by Hesketh *et al.* (1983). Phosphoinositide degradation and Ca^{2+} mobilization result from cross-linking of cell surface receptors, and are presumed to be the initial signalling events in cell activation. The present results and those of Hesketh *et al.* (1983) therefore suggest that high concentrations of Con A inhibit a later step in thymocyte proliferation.

Effects of cyclosporine on inositol phosphate formation in B cells and thymocytes

In the experiments summarized in Fig. 2, thymocytes were stimulated with an optimally mitogenic concentration of Con A in the presence of varying doses of CS. Proliferation, estimated from [^{3}H]thymidine

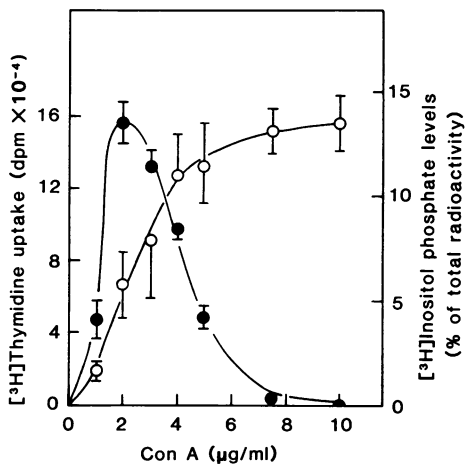

Figure 1. Dose-dependence of Con-induced inositol phosphate accumulation and DNA synthesis in mouse thymocytes. Aliquots of 3×10^6 [^{3}H]inositol-labelled thymocytes, preincubated for 2 hr in medium containing 5 mM LiCl, received Con A to final concentrations of 0–10 μg/ml. [^{3}H]inositol phosphate levels (○) were determined after 2 hr. Unlabelled thymocytes were cultured in microtitre wells (10^6 cells/well) with 0–10 μg Con A/ml, and [^{3}H]thymidine incorporation (●) in these cells was determined 3 days later. Proliferation and inositol phosphate accumulation were studied with cells from the same preparations. [^{3}H]inositol phosphate levels are expressed as percentages of the total cellular radioactivity (78,000 d.p.m. on average) and are corrected for levels in unstimulated cells ($1{\cdot}1 \pm 0{\cdot}1\%$). [^{3}H]thymidine uptakes are given as d.p.m./well. Points are means ± SEM or five to eight replicates from two separate experiments.

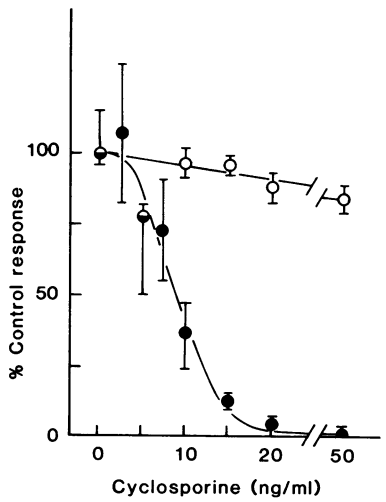

Figure 2. Effects of CS on DNA synthesis and inositol phosphate accumulation in Con A-stimulated thymocytes. Aliquots of 3×10^6 [^{3}H]inositol-labelled thymocytes, preincubated for 1·5 hr in medium with 5 mM LiCl, received 0–50 ng CS/ml. After a further 30 min, the cells were stimulated by adding Con A to 2 μg/ml. [^{3}H]inositol phosphate levels (○) were determined 2 hr later. Further aliquots of 10^6 unlabelled thymocytes in mirotitre wells were preincubated for 30 min with CS, and then received Con A to 2 μg/ml. [^{3}H]thymidine incorporation (●) in these cells was determined 3 days later. [^{3}H]inositol phosphate levels, corrected for levels in unstimulated cells, are expressed as percentages of the level at 0 ng CS/ml ($9{\cdot}7 \pm 2{\cdot}1\%$ of the total cellular radioactivity). [^{3}H]thymidine uptakes are expressed as percentages of the control response (no CS added: 122,000 d.p.m. on average). Points are means ± SEM of four to seven replicates from two separate experiments. Cyclosporine solvent alone (1 p.p.m. Tween 80 and 4 p.p.m. ethanol) did not effect either response.

uptake, was suppressed by low doses of CS (50% inhibition at 5–10 ng/ml). Inositol phosphate formation, however, was unaffected by CS at concentrations up to 50 ng/ml.

B cells were stimulated with a mitogenic concentration (50 μg/ml) of anti-Ig in the presence of varying doses of CS (Fig. 3). As in thymocytes, low doses of CS effectively inhibited proliferation (50% inhibition at CS < 5 ng/ml), whereas inositol phosphate formation was unaffected. In further experiments, we found that even 300 ng CS/ml did not suppress anti-Ig-induced inositol phosphate formation (data not shown).

In the experiments shown in Figs 2 and 3, the cells were stimulated after 30 min of preincubation with CS. This period should be sufficient to allow the lipophilic drug to penetrate the cells. However, to ensure that these results do not simply reflect slow penetration of the drug, we studied phosphoinositide degradation in B cells and thymocytes after 6 hr of preincubation with 50 ng CS/ml: even under these conditions, mitogen-stimulated inositol phosphate formation was unaffected.

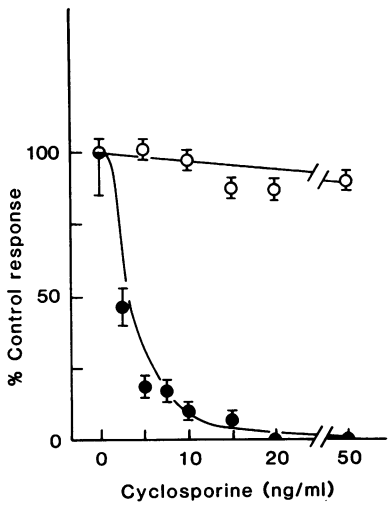

Figure 3. Effects of CS on DNA synthesis and inositol phosphate accumulation in anti-Ig stimulated B cells. Aliquots of 3×10^6 [^{3}H]inositol-labelled B cells were preincubated for 30 min with 0–50 ng CS/ml in medium containing 5 mM LiCl. Then, the cells were stimulated by adding anti-Ig to 50 μg/ml, and [^{3}H]inositol phosphate levels (○) were determined 1 hr later. Further aliquots of 10^6 unlabelled B cells in microtitre wells were preincubated for 30 min with 0–50 ng CS/ml, and then received anti-Ig to 50 μg/ml. [^{3}H]thymidine incorporation (●) in these cells was determined 3 days later. [^{3}H]inositol phosphate levels, corrected for levels in unstimulated cells, are expressed as percentages of the level in the absence of CS (21·1 ± 0·9% of the total cellular radioactivity). [^{3}H]thymidine uptakes are expressed as percentages of the control response (no CS added: 20,000 d.p.m.). Points are means ± SEM of three to four replicates from one experiment. Tween-ethanol solvent alone did not effect either response.

DISCUSSION

Our study confirms that low levels of CS abrogate the proliferative responses of thymocytes and B cells to Con A and anti-Ig, respectively. However, the drug did not have an appreciable effect on the induction of PIP_2 degradation by these mitogens. Since this response is believed to be involved in 'second messenger' generation (Berridge, 1984a,b), our results strongly suggest that CS does not interfere with early intracellular signalling following ligation of antigen receptors on B cells (and probably those on T cells as well).

Inositol trisphosphate (IP_3), a primary product of PIP_2 breakdown, has been shown to release Ca^{2+} from intracellular stores in various cell types (Berridge, 1984a). However, an early increase in $[Ca^{2+}]_i$ following receptor ligation does not necessarily reflect preceding PIP_2 degradation (Fisher, Bakshian & Baldassare, 1985; Volpi *et al.*, 1984). It has been demonstrated that incubation of B cells with anti-Ig or thymocytes with Con A leads to increases in $[Ca^{2+}]_i$ that are mainly due to influx of Ca^{2+} from the exterior (Pozzan *et al.*, 1982; Hesketh *et al.*, 1985). This suggests that, apart from provoking Ca^{2+} release from internal stores, anti-Ig and Con A activate yet another mechanism for increasing $[Ca^{2+}]_i$. since CS does not block mitogen-stimulated IP_3 release, it presumably does not affect Ca^{2+} release from internal stores. Metcalfe (1984) showed that CS does not diminish increases in $[Ca^{2+}]_i$ in Con A-stimulated thymocytes, which indicates that the drug does not abrogate Ca^{2+} influx either.

The lipophilic nature of CS suggests that it may exert its action by interfering with membrane-associated events. Earlier work by Ryffel *et al.* (1980, 1982) showed that CS binds with high affinity to human and murine lymphocytes. Binding was rapidly reversible, suggesting that, after binding, CS remains associated with the plasma membrane. These findings were confirmed by LeGrue, Friedman & Kahan (1983), but they additionally demonstrated similar binding of CS to phospholipid vesicles. This suggested that, rather than binding to a plasma membrane receptor, CS partitions into the membrane lipid phase. However, results from other workers point to a possible intracel-

lular site of action of CS. Handschuhmacher *et al.* (1984) have isolated and characterized a cytosolic protein that tightly binds CS. The protein, called cyclophilin, has been detected in thymocytes from various species and in mature T cells. It is also found in non-lymphoid tissues, with high concentrations in brain and kidney—organs prone to the toxic side effects of CS. As yet, nothing is known about the function of cyclophilin. However, it has recently been shown that calmodulin binds CS and that calmodulin-specific antibodies cross-react with cyclophilin, thus suggesting a relationship between the two proteins (Colombani, Robb & Hess, 1985). Recent data suggest that, in T cells, CS blocks an event preceeding transcription (or actual transcription) of the T-cell growth factor (IL-2) gene (Kronke *et al.*, 1984; Granelli-Piperno, Inaba & Steinman, 1984). However, since these studies were done with already proliferating T cells, it is unclear how they relate to the inhibitory effects of the drug on activation of resting lymphocytes.

In conclusion, it is still unclear how and where CS inhibits proliferation of lymphocytes. Susceptiblity to inhibition by CS appears to be restricted to those lymphocyte activators that induce PIP_2 degradation and Ca^{2+} mobilization (Bijsterbosch *et al.*, 1985; Kay *et al.*, 1983b). Stimulation of both pig and mouse lymphocytes by Ca^{2+} ionophores is CS-sensitive as well (Kay *et al.*, 1983a; Klaus, Bijsterbosch & Holman, 1985). Activation by agents such as lipopolysaccharide and phorbol esters, which stimulate lymphocytes without increasing Ca^{2+} or enhancing PIP_2 degradation, is CS-resistant (Bijsterbosch *et al.*, 1985; Kay *et al.*, 1983b). Mitogens that elicit PIP_2 degradation and Ca^{2+} mobilization probably mimic the effects of specific antigen, and it is believed that these two events are the initial signals for the induction of cell growth. However, it is now clear that PIP_2 degradation and Ca^{2+} mobilization themselves are not affected by CS. The drug, therefore, presumably interferes with a step further along the putative pathway initiated by the two events. The recently reported binding of CS to calmodulin (Colombani *et al.*, 1985) is in line with this concept.

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