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# Opposing effects of rapamycin and cyclosporin A on activation-induced $Ca^{2+}$ release

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**Abstract**

Insofar as $Ca^{2+}$ plays a major role in T cell activation, we investigated the effect of the immunosuppressants cyclosporin A and rapamycin on T cell proliferation and on the activation-induced increase in $[Ca^{2+}]_i$. Both cyclosporin A and rapamycin inhibited mitogen (concanavalin A and phytohemagglutinin) and ionomycin + phorbol myristate acetate (PMA)-driven T cell proliferation ($Ca^{2+}$-dependent). However, only rapamycin suppressed T cell proliferation stimulated by anti-CD28 antibody (Ab) + PMA, and recombinant interleukin-6-stimulated proliferation of the interleukin-6 dependent B9 cells ($Ca^{2+}$-independent). These differences were associated with a different effect of both drugs on $Ca^{2+}$ release, as cyclosporin A attenuated while rapamycin augmented the mitogen-induced elevation in $[Ca^{2+}]_i$. Collectively, this supports the notion that $Ca^{2+}$ is required in early stages of T cell activation, and that cyclosporin A blocked only $Ca^{2+}$-dependent while rapamycin blocked both $Ca^{2+}$-dependent and -independent events of T cell activation. 

*Keywords:* Cyclosporin A; Rapamycin; $Ca^{2+}$; T cells; EGTA (ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid

## 1. Introduction

T cell activation through the T cell receptor/CD3 complex in conjunction with appropriate costimulation involves rapid phosphorylation of several intra-cellular molecules by src-like protein tyrosine kinases, and results in the activation of protein kinase C, elevation of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$), and induction of the interleukin-2 autocrine pathway. An orderly expression of cytokine genes and their high-affinity receptors ensues, followed by induction of cellular proliferation (Almawi et al., 1998). This $Ca^{2+}$-dependent phase of T cell activation is followed by a $Ca^{2+}$-independent phase, as exemplified by the interaction of T cell-bound CD28/CTLA-4 with accessory cell-bound B7.1/B7.2 (June et al., 1987), or by the interaction of cytokines with their high-affinity receptors.

Cyclosporin A and rapamycin are potent immunosuppressive drugs that exert their effects by inhibiting distinct events of the T cell activation cascade (Halloran, 1996), but synergize in inhibiting T cell immunity (Wasowska et al., 1997). Both drugs act by binding to their cytosolic receptors. Cyclosporin A binds cyclophilin (Almawi et al., 1993), thereby antagonizing calcineurin activity and hence interrupting signal transduction events that link T cell receptor engagement to induction of cytokine expression (Emmel et al., 1989). Rapamycin, by binding to the FK506-binding protein of 12 Mr kDa (FKBP12; Sabers et al., 1995), antagonizes biochemical events associated with cytokine-stimulated signaling events or those associated with cross-linking of the CD28 homodimer (Almawi et al., 1993).

In view of the central role of $Ca^{2+}$ in driving optimal T cell activation, and the different requirement for $Ca^{2+}$ at distinct stages of T cell activation, we investigated the effects of cyclosporin A and rapamycin on $Ca^{2+}$-dependent and -independent proliferative responses and on the activation driven increase in $[Ca^{2+}]_i$. Results obtained clearly support the notion that cyclosporin A blocks the $Ca^{2+}$-dependent response, whereas rapamycin blocks both $Ca^{2+}$-dependent and -independent events of T cell activation.

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## 2. Materials and methods

### 2.1. Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were prepared from the venous blood of healthy volunteers by hypaque-ficoll (sg 1.076) sedimentation (Pharmacia Fine Chemicals, Dorval, Canada). The interphase containing peripheral blood mononuclear cells was suspended in RPMI-1640 culture medium supplemented with 10% human AB serum (Whittaker, Bethesda, MD), 2 mM L-glutamine (Gibco BRL, Mississauga, Canada), and penicillin–streptomycin (Gibco BRL) at 100 IU/ml and 100 μg/ml, respectively. This medium is referred to thereafter as ''complete medium''. Cell viability was determined by the Trypan blue exclusion method.

### 2.2. Reagents and drugs

Phorbol myristate acetate (PMA), concanavalin A, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and ionomycin were obtained from Sigma (St. Louis, MO), and phytohemagglutinin was purchased from Difco Laboratories (Detroit, MI). Cyclosporin A (Sandoz, Basel, Switzerland) and rapamycin (Wyeth-Ayerst; Princeton, NJ) were prepared as 10 mM stocks in 70% ethanol; further dilutions were made with RPMI-1640. The anti-CD28 antibody (Ab), 9.3, was generously provided by Bristol-Myers-Squibb (Philadelphia, PA), and the interleukin-6 sensitive cell line, B9, was kindly supplied by Dr. L. Aarden (Amsterdam, the Netherlands). Recombinant IL-6 was kindly donated by Dr. Steven Gillis (Immunex, Seattle, WA).

### 2.3. Proliferation assay

Peripheral blood mononuclear cells ($10^6$ cells/ml) were cultured in 96-well flat-bottom microtiter plates, treated with drugs or controls, stimulated and incubated for 72 h at 37°C. B9 cells ($2 \times 10^5$ cells/ml) were similarly cultured and treated, stimulated with recombinant interleukin-6 (200 U/ml), and incubated for 48 h at 37°C. [$^3$H]Thymidine (Dupont-NEN, Mississauga, Canada) was added to the plates (1 μCi/well) 4 h prior to termination of culture; cellular proliferation (in counts per minute; CPM) was quantitated by liquid scintillation.

### 2.4. Measurement of $[Ca^{2+}]_i$ levels

$[Ca^{2+}]_i$ levels were determined by flow cytometry on FACStar Plus (Becton-Dickinson, Toronto, Canada), using

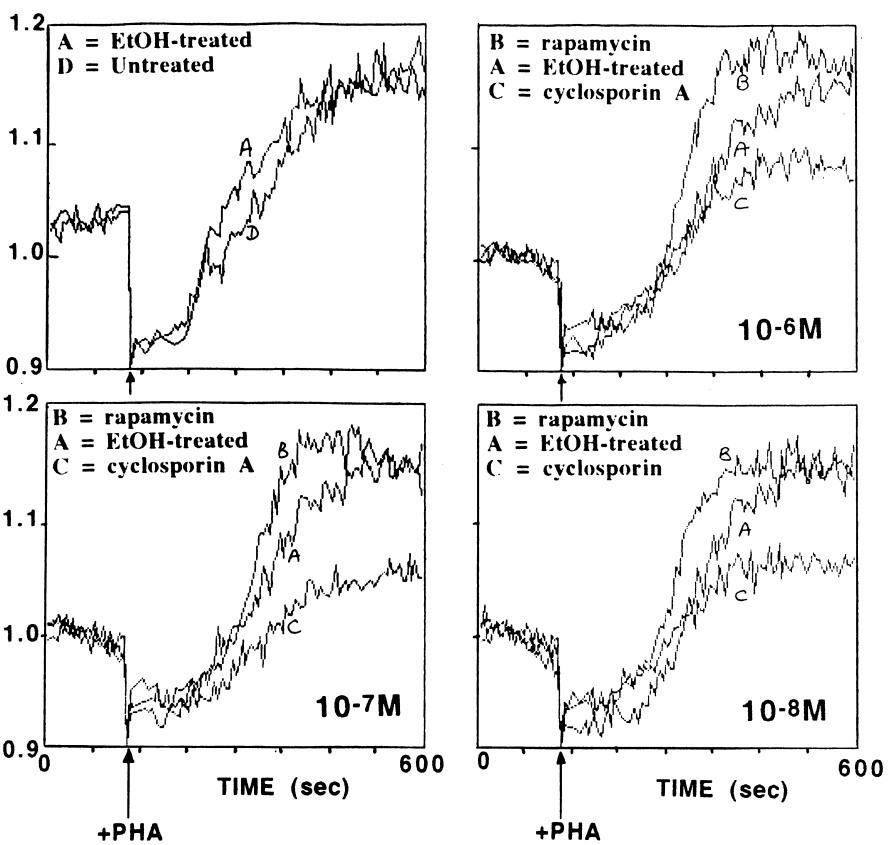


Fig. 1. Calcium mobilization in response to phytohemagglutinin stimulation. Medium: ethanol (A), rapamycin (B), and cyclosporin A (C) pretreated and Indo-1AM-loaded peripheral blood mononuclear cells were stimulated with phytohemagglutinin (25 μg/ml); changes in $[Ca^{2+}]_i$ were determined by flow cytometry.

the acetoxymethylester of indo-1 (Indo-1AM) (Sigma) as indicator dye. Briefly, peripheral blood mononuclear cells ($10^6$ cells/ml) were treated with drugs or vehicle for the indicated time periods and loaded with Indo-1AM (10 μg/ml) in the dark for 45 min at 37°C. The cells were then washed, resuspended at $10^7$ cells/ml, and stimulated with phytohemagglutinin (25 μg/ml). $[Ca^{2+}]_i$ levels were measured by recording the shift in fluorescence (excitation wavelength, 339 nm; emission wavelength, 492 nm) before and after phytohemagglutinin addition over a 10-min period. The change in $Ca^{2+}$ concentrations was calculated as described by Muthukkumar et al. (1993).

## 3. Results

### 3.1. Effect of cyclosporin A and rapamycin on $Ca^{2+}$ release

Peripheral blood mononuclear cells were pretreated for 4–6 h with ethanol (solvent), cyclosporin A or rapamycin at the indicated concentrations, preloaded with Indo-1AM in the dark, and stimulated with the mitogen phytohemagglutinin. Changes in $[Ca^{2+}]_i$ were recorded 2.2 min before (baseline) and over a 7.8-min period after mitogen addition. Mitogen-induced stimulation resulted in a rapid rise in $[Ca^{2+}]_i$ which was maximal 4.8 min after mitogen addition (Fig. 1). Whereas, cyclosporin A attenuated the mitogen-stimulated rise in $[Ca^{2+}]_i$, rapamycin augmented it (Fig. 1), as compared to $[Ca^{2+}]_i$ levels in medium- or ethanol-pretreated and stimulated cultures.

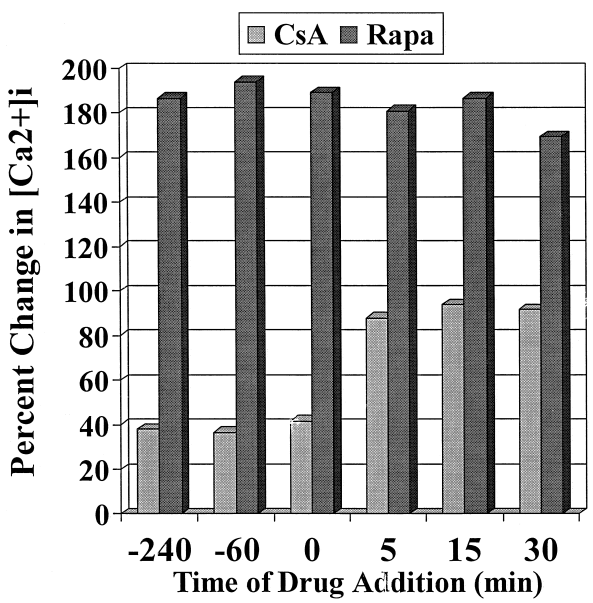


Fig. 2. Kinetics of cyclosporin A and rapamycin action. Percent change in $[Ca^{2+}]_i$ in peripheral blood mononuclear cells treated with cyclosporin A and rapamycin at 1 μM each at the indicated time before or after phytohemagglutinin (25 μg/ml) addition relative to the $[Ca^{2+}]_i$ change in ethanol-pretreated cultures. Results shown represent the mean of four experiments. $[Ca^{2+}]_i$ levels (mean ± S.E.M.), resting (non-stimulated) cells, 124 ± 22 nM, phytohemagglutinin-activated cells, 295 ± 32 nM.

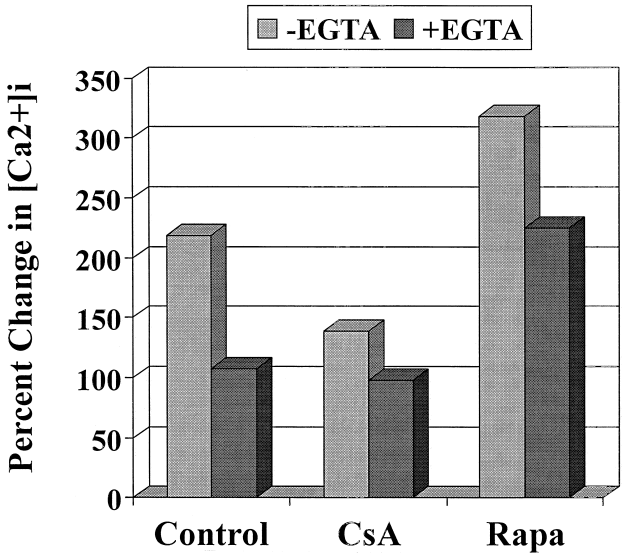


Fig. 3. Effect of cyclosporin A and rapamycin on $[Ca^{2+}]_i$ in the presence or absence of EGTA. Percent change in $[Ca^{2+}]_i$ in PBML pretreated for 4 h with cyclosporin A (CsA) and rapamycin (Rapa) (at 1 μM each) or a corresponding volume of ethanol (Control), loaded with Indo-1AM, and stimulated with phytohemagglutinin in the presence (+EGTA) or absence (−EGTA) of the calcium chelator, EGTA (5 mM). Results shown are the mean of six individually performed experiments.

The inhibitory and stimulatory effects of cyclosporin A and rapamycin, respectively, on mitogen-stimulated $Ca^{2+}$ release were seen at concentrations as low as 1 nM for each drug, reaching a maximum at 1 μM or higher. It is noteworthy that neither drug was capable of altering basal (non-stimulated) $Ca^{2+}$ concentrations, and that cyclosporin A, while clearly attenuating the activation-induced rise in $[Ca^{2+}]_i$, did not abolish it completely (Fig. 1).

### 3.2. Kinetics of cyclosporin A and rapamycin action

The time dependency of cyclosporin A and rapamycin action in attenuating and augmenting $[Ca^{2+}]_i$, respectively, was next examined. Cyclosporin A and rapamycin, each at a final concentration of 1 μM, were added before, at, or after mitogen addition to Indo-1AM-loaded cultures; percent changes in $[Ca^{2+}]_i$ were recorded over a 10-min period after their addition, and were made in reference to control cultures treated with a corresponding volume of ethanol. Cyclosporin A attenuated the activation-stimulated rise in $[Ca^{2+}]_i$ only if added before or at the same time as phytohemagglutinin; its addition after mitogen stimulation was ineffective (Fig. 2). In contrast, rapamycin augmented the mitogen-stimulated rise in $[Ca^{2+}]_i$ even if added after activation (Fig. 2).

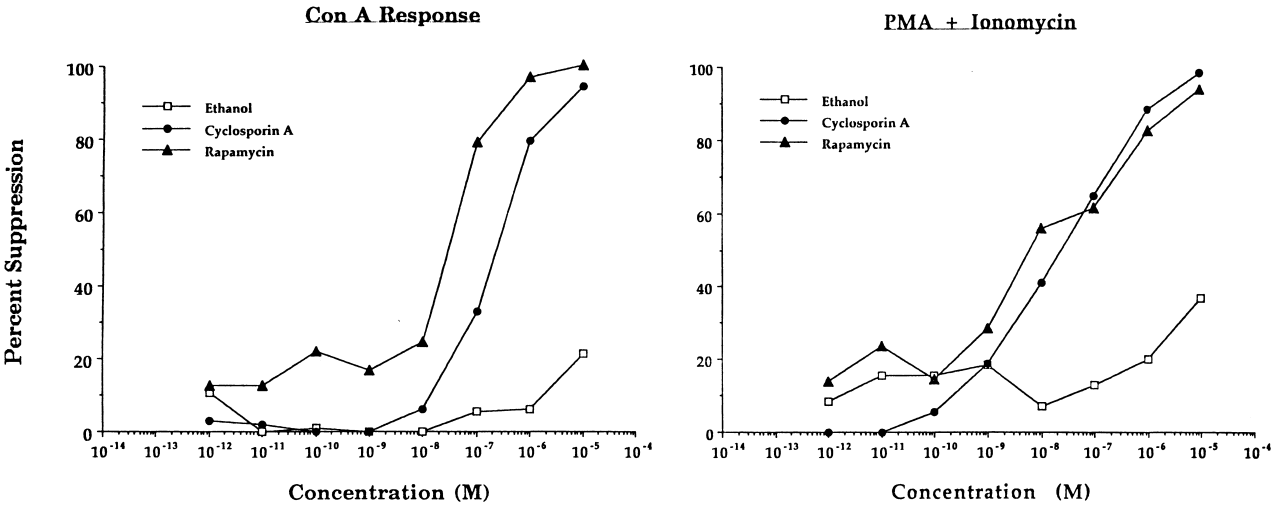


Fig. 4. Suppression of $Ca^{2+}$-dependent proliferative responses by cyclosporin A and rapamycin. Percent suppression of T cell proliferation stimulated by concanavalin A (5 μg/ml; left), or PMA (5 ng/ml) + ionomycin (0.1 μM) (right). Results shown are the means of eight individually performed experiments. Proliferation values (in CPM): background, 128 ± 18; Concanavalin A, 67790 ± 12593; PMA + ionomycin, 82486 ± 12043. Percent suppression was calculated as per: {1 − [(test-background)/(control-background)]} × 100%.

### *3.3. Effect of $Ca^{2+}$ chelation on cyclosporin A and rapamycin effects on $[Ca^{2+}]_i$*

We next determined whether the attenuation and augmentation of the mitogen-stimulated rise in $[Ca^{2+}]_i$ mediated by cyclosporin A and rapamycin, respectively, was dependent on extracellular $Ca^{2+}$ influx. Indo-1AM-loaded cultures treated with cyclosporin A, rapamycin, or a corresponding volume of ethanol (Control) were treated with the $Ca^{2+}$ chelator, EGTA (5 mM; +EGTA) or control (−EGTA), and stimulated with phytohemagglutinin. Results from Fig. 3 show that EGTA completely abolished the stimulated rise in $[Ca^{2+}]_i$, in cyclosporin A-treated cultures. It was of interest to find that EGTA only partially suppressed the mitogen-induced rise in $[Ca^{2+}]_i$ in rapamycin-treated cultures (Fig. 3), an indication that the rapamycin-augmented rise in $[Ca^{2+}]_i$ follows both $Ca^{2+}$-dependent and -independent pathways.

### *3.4. Effect of cyclosporin A and rapamycin on proliferative responses*

Insofar as T cell activation follows $Ca^{2+}$-dependent and -independent pathways, and as cyclosporin A and rapamycin differentially affected the activation-induced rise in $[Ca^{2+}]_i$, we next assessed the effect of cyclosporin A

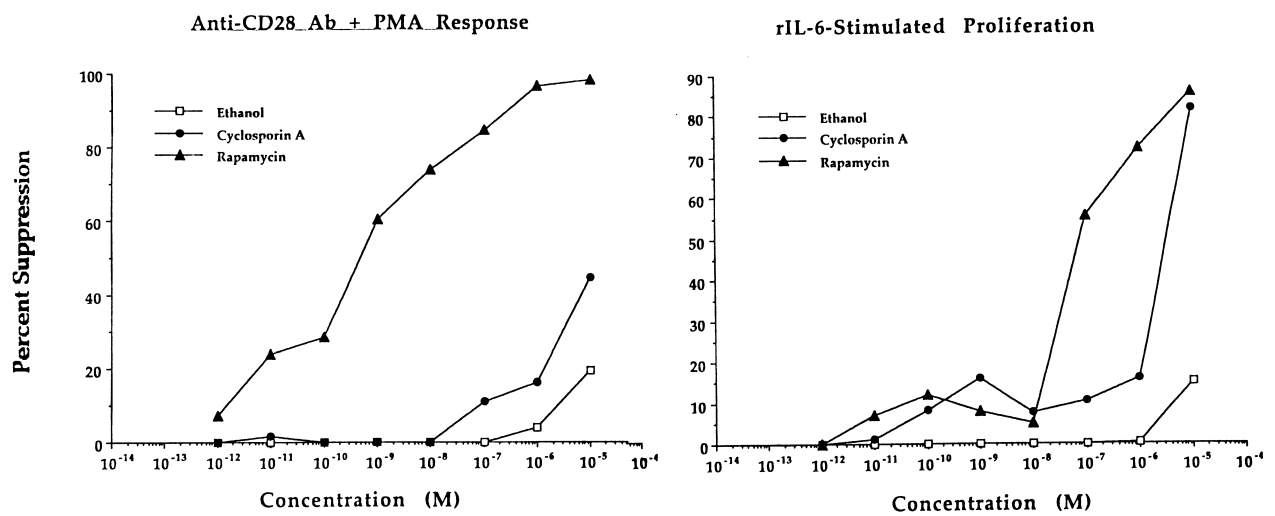


Fig. 5. Suppression of $Ca^{2+}$-independent proliferative responses by cyclosporin A and rapamycin. Percent suppression of T cell proliferation stimulated by cross-linking anti-CD28 Ab (1:200) + PMA (5 ng/ml) (left), and recombinant interleukin-6-driven proliferation of B9 cells (right). Shown is the mean of eight (PBML) or four (B9 cells) individually performed experiments. Proliferation values (in CPM): peripheral blood mononuclear cells: background, 1291 ± 404; anti-CD28 Ab + PMA, 73011 ± 5948; B9 cells: background, 217 ± 51; rIL-6, 20125 ± 1585. Percent suppression was calculated as per Fig. 4.

and rapamycin on $Ca^{2+}$-dependent and independent T cell proliferation. The former was elicited by the mitogens phytohemagglutinin and concanavalin A, and the ''CD3-bypass'' stimulation regimen, PMA + ionomycin. The latter was achieved by cross-linking of T cell bound CD28 with the anti-CD28 Ab, 9.3 in conjunction with PMA, and by stimulation of the interleukin-6 dependent cell line, B9, with recombinant interleukin-6.

Whereas, both cyclosporin A and rapamycin, in a concentration-dependent manner, inhibited both concanavalin A and PMA + ionomycin ($Ca^{2+}$-dependent) proliferative responses (Fig. 4), only rapamycin blocked anti-CD28 Ab + PMA T cell and recombinant interleukin-6-driven B9 cell ($Ca^{2+}$-independent) proliferation (Fig. 5). The inhibitory effect of cyclosporin A on recombinant interlukin-6 stimulated proliferation of B9 cells seen at 10 μM or higher concentrations reflects a non-specific cytotoxic effect, as this was associated with a significant reduction in cell viability (data not shown).

## 4. Discussion

The results reported here demonstrate that cyclosporin A blocked $Ca^{2+}$ dependent whereas rapamycin blocked both $Ca^{2+}$-dependent and -independent proliferative responses, and that cyclosporin A attenuated while rapamycin augmented the activation-induced rise in $[Ca^{2+}]_i$. Although the inhibitory effect of cyclosporin A on $Ca^{2+}$-dependent T cell effector function (i.e., proliferation and interleukin-2 secretion) is well established, its effect on the T cell activation-induced rise in $[Ca^{2+}]_i$ is controversial, as some reports described an inhibitory (Draberova, 1990), stimulatory (Damjanovich et al., 1987), or no effect (Harris et al., 1987; Muthukkumar et al., 1993) of cyclosporin A on $[Ca^{2+}]_i$ . In these studies, the effect of cyclosporin A was assessed in cloned cytotoxic T lymphocytes (Harris et al., 1987), anti-immunoglobulin M (IgM)-stimulated immature B cells (Muthukkumar et al., 1993), mast cells (Draberova, 1990), or under highly artificial conditions (Damjanovich et al., 1987). While not negating earlier findings, it appears that the effect of cyclosporin A on $[Ca^{2+}]_i$ depends on the cell type investigated, and the experimental conditions utilized.

While the effect of cyclosporin A on $Ca^{2+}$-dependent proliferation and the rise in $[Ca^{2+}]_i$ were not surprising in light of its effect on $Ca^{2+}$-dependent events of T cell activation (activation of calcineurin, induction of cytokine gene expression) (Emmel et al., 1989), it was of interest — and the first demonstration in human lymphocytes — that rapamycin augmented the $[Ca^{2+}]_i$ rise elicited in mitogen- (Fig. 1) and anti-CD3 Ab-stimulated (data not shown) human peripheral blood mononuclear cells. While it clearly augmented the activation-induced rise in $[Ca^{2+}]_i$, rapamycin apparently did not affect basal (non-stimulated) $Ca^{2+}$ levels, which suggested that other ''activation-associated'' signals must cooperate with rapamycin in stimulating an increase in $[Ca^{2+}]_i$ . Furthermore, cyclosporin A acted proximally since its addition after mitogen stimulation abolished its attenuation of the activation-induced rise in $[Ca^{2+}]_i$. In contrast, rapamycin appeared to act more distally since its introduction (even 30 min post-activation) resulted in a significant rise in $[Ca^{2+}]_i$.

This was further shown in the EGTA studies, where chelation of extracellular $Ca^{2+}$ partially abolished the rapamycin-mediated augmentation in $[Ca^{2+}]_i$, which was unexpected since EGTA was previously shown, and confirmed here, to abolish the mitogen-stimulated increase in $[Ca^{2+}]_i$ (Watman et al., 1988; Komada et al., 1996). However, mitogen-stimulated proliferation requires other signals imparted by accessory cells in addition to extracellular $Ca^{2+}$. In reducing the availability of $[Ca^{2+}]_i$, EGTA may have affected early proximal events of T cell activation, without rendering the cells anergic (Watman et al., 1988). Accordingly, activation of intracellular $Ca^{2+}$ pools directly by rapamycin (in conjunction with other signals) stimulated an increase in $[Ca^{2+}]_i$ that was independent of extracellular $Ca^{2+}$ (i.e., ''$Ca^{2+}$-evoked $Ca^{2+}$ release''). This was supported by studies which demonstrated that, by binding FKBP-12, rapamycin destabilizes its tight association with $[Ca^{2+}]_i$-release channels (ryanodine receptors or IP3 receptors) in striated (Ahern et al., 1997) and non-striated (Graves et al., 1997; Marks, 1997) cells, resulting in significant accumulation of $[Ca^{2+}]_i$ (Ahern et al., 1997; Marks, 1997).

Mechanistically, cyclosporin A blocks T cell activation specifically by antagonizing calcineurin activity (Takahashi et al., 1989), thus preventing the correct import of nuclear factors required for optimal transcription of interleukin-2 and other cytokine genes (Emmel et al., 1989). Rapamycin acts later in the T cell activation pathway, and its target of action appears to be ribosomal p70S6 kinase, a key $Ca^{2+}$-regulated enzyme involved in signaling via cytokine and growth factor receptors (Calvo et al., 1992; Graves et al., 1997). Thus, by differentially affecting the activation-induced rise in $[Ca^{2+}]_i$, cyclosporin A and rapamycin selectively affected distinct events of T cell activation whereby cyclosporin A blocked only $Ca^{2+}$-dependent, while rapamycin blocked both $Ca^{2+}$-dependent and $Ca^{2+}$-independent T cell proliferation. The significance of the rapamycin-mediated potentiation of the T cell activation-induced rise in $[Ca^{2+}]_i$ on its anti-proliferative effect remains to be determined.

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