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# FK-506 AND CYCLOSPORIN A: SELECTIVE INHIBITION OF CALCIUM IONOPHORE-INDUCED POLYMORPHONUCLEAR LEUKOCYTE DEGRANULATION

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**Abstract**—This paper investigates the abilities of FK-506 and cyclosporin A (CsA) to inhibit human polymorphonuclear leukocyte (PMNL) degranulation. PMNLs, purified from human blood, were stimulated *in vitro* with A23187, ionomycin, the complement derived peptide C5a, formylmethionylleucinylphenylalanine (FMLP) or phorbol myristate acetate (PMA). Degranulation was assessed by measuring the release of either lactoferrin or *N*-acetyl-β-D-glucosaminidase (NAG). Both FK-506 and CsA produced a concentration-related inhibition of degranulation induced by either A23187 or ionomycin but did not affect C5a-, FMLP- or PMA-induced degranulation. The $IC_{50}$ values for inhibition of degranulation (approximately 0.7 nM for FK-506 and 33.7 nM for CsA) are very close to the published values for inhibition of human T-cell proliferation. Removal of calcium from the incubation medium with ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) totally inhibited calcium ionophore-induced degranulation but had no effect against C5a-, FMLP- or PMA-induced degranulation. Preincubation of PMNLs with actinomycin D or cycloheximide did not affect either A23187- or PMA-induced degranulation. Non-immunosuppressive analogs of CsA were ineffective at inhibiting degranulation. Rapamycin, a macrolide structurally related to FK-506, did not inhibit degranulation but it did antagonize the inhibition produced by FK-506. Given the similar profiles of activity of FK-506 and CsA in neutrophils and T cells, we conclude that similar activation or signal transduction pathways may be present in both T cells and neutrophils. Because A23187-induced PMNL degranulation was not sensitive to either actinomycin D or cycloheximide, it is apparent that the signal transduction pathways ultimately control different cellular functions.

Cyclosporin A (CsA†), a cyclic peptide, and FK-506, a neutral macrolide, are potent immunosuppressive agents. Cyclosporin A has been in use clinically for over 10 years to prevent allogeneic graft rejection [1, 2], whereas FK-506 has only recently undergone limited clinical trials for use in the prevention of graft rejection following allogeneic liver transplantation [3]. Both compounds achieve immunosuppression via inhibition of the transcription of a restricted number of T-cell activation genes [4–6]; however, the precise mechanism by which this occurs is unknown.

Distinct binding proteins for CsA [7] and for FK-506 [8] have been identified, prompting the hypothesis that the binding of immunosuppressants to these proteins may relate to their immunosuppressive activity. It has been discovered recently that the CsA binding protein, cyclophilin, and the FK-506 binding protein, FKBP, share a common property of catalyzing the *cis-trans* isomerization of peptidyl proline bonds [9–11]. The binding proteins are widely distributed within the tissues of an animal and are highly conserved across different animal species [12, 13]. It is, however, something of an enigma that despite the ubiquitous distribution of the binding proteins both CsA and FK-506 are highly selective in acting on lymphocytes, particularly T-helper cells. The selective action on lymphocytes may be explained on the basis of a requirement for a particular activation signal or a particular signal transduction pathway in response to an activation signal. This proposal is derived from the observations that in T-lymphocytes only those stimuli which produce an increase in intracellular calcium are sensitive to the effects of either CsA or FK-506 [14, 15].

For the most part CsA is not reported to affect polymorphonuclear leukocyte (PMNL) or monocyte activation in response to a wide variety of stimuli including formylmethionylleucinylphenylalanine (FMLP), zymosan activated plasma, phorbol myristate acetate (PMA) or opsonized zymosan [16–18]. There is a report that CsA does reduce calcium ionophore (A23187) induced lactoferrin release from human PMNLs [17]. Although the concentration of CsA used in these experiments, 4 μM was considerably greater than that required for immunosuppression *in vitro*, the effect was specific in that degranulation induced by zymosan or PMA was not reduced. It has also been reported that CsA, at pharmacologically relevant concentrations, will

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† Abbreviations: CsA, cyclosporin A; PMNL, polymorphonuclear leukocyte; FMLP, formylmethionylleucinylphenylalanine; PMA, phorbol myristate acetate; and NAG, *N*-acetyl-β-D-glucosaminidase.

inhibit the release of histamine, serotonin and leukotrienes from human basophils, rat mast cells and rat basophilic leukemia cells in response to A23187, anti-IgE and, to a lesser extent, in response to specific antigen challenge or to concanavalin A [19–22]. Thus, the specificity of CsA may not be for a particular cell type but to an activation signal or signal transduction pathway whose distribution is not restricted to T cells.

We have confirmed and extended the observations of an effect of CsA on A23187-induced lactoferrin release from neutrophils by comparing the inhibitory activities of both CsA and FK-506 against PMNL degranulation induced by different agonists. In this paper we show that both immunosuppressive agents were effective inhibitors of PMNL degranulation at concentrations similar to those required for immunosuppression. The inhibitory activity was restricted to degranulation induced by calcium ionophores. We further show that rapamycin, an immunosuppressive agent structurally similar to FK-506, and non-immunosuppressive analogs of CsA failed to inhibit degranulation. Rapamycin reversed the inhibitory effect of FK-506 on PMNL degranulation similar to its reported action on T-cell activation.

## MATERIALS AND METHODS

*Reagents.* A23187, FMLP, PMA, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, actinomycin D and cycloheximide were obtained from the Sigma Chemical Co., St. Louis, MO. Ionomycin was obtained from Calbiochem, San Diego, CA. FK-506 and rapamycin were obtained from Dr. R. Borris and CsA was provided by Dr. P. Durette, both of Merck Sharp & Dohme Research Laboratories. FK-506 was isolated from a culture of *Streptomyces tsukubaensis* derived from the Fujisawa Pharmaceutical Company's (Osaka, Japan) deposit to the Danish patent office. Rapamycin was isolated according to previously published procedures [23] from *Streptomyces hydroscopicus* cultures deposited with the ATCC. FK-506, CsA and rapamycin were prepared as 1 mg/mL stock solutions in ethanol and stored at −20°; they were diluted into Hanks' Balanced Salt Solution (HBSS) immediately prior to use. Recombinant human C5a was provided by Dr. M. S. Springer of Merck Sharp & Dohme Research Laboratories.

*Cells.* PMNLs were isolated from freshly drawn heparinized (10 units/mL) human venous blood. Blood was gently mixed with an equal volume of Dulbecco's $Ca^{2+}/Mg^{2+}$-free phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY), and then 30 mL was layered on top of 20 mL of lymphocyte separation medium (Organon, Durham, NC) in 50-mL polypropylene tubes. The tubes were centrifuged at 800 *g* for 25 min at 25°. The upper layers of plasma and mononuclear cells were discarded and the pellet of red blood cells and PMNLs were resuspended in a red cell lysing solution (155 mM $NH_4Cl$; 0.1 mM $K_2EDTA$; 10 mM $KHCO_3$) at 4°. The suspension was centrifuged immediately at 500 *g* for 10 min at 4°. The lysing procedure was repeated until all red cells had been removed. The remaining cells (>99% PMNL: > 99% viable by Trypan blue exclusion) were washed in 50 mL of Dulbecco's $Ca^{2+}/Mg^{2+}$-free PBS containing 1 mg/mL of bovine serum albumin (BSA; Sigma) and finally resuspended in HBSS (GIBCO) containing 25 mM HEPES buffer (GIBCO) and 5 mg/mL BSA.

*Cell incubations.* Different concentrations of FK-506, CsA or rapamycin were added to 0.5-mL suspensions of PMNL ($5 \times 10^6$/mL) in polypropylene tubes at room temperature for 25 min. Incubations were performed at room temperature because at 4° the entry of either CsA [7] or FK-506 [8] into cells is minimal, whereas at 37° the PMNLs have a tendency to clump. In a preliminary experiment, slightly less inhibition of degranulation was observed using a 15-min incubation. At the end of the incubation period, 10 μL of cytochalasin B (Sigma) was added to all cell suspensions to give a final concentration of 5 μg/mL and the cells were incubated for a further 5 min at 37°. One hundred microliter aliquots in triplicate were added to round bottom 96-well microtitre plates (COSTAR, Cambridge, MA) containing 100 μL of appropriate stimuli and which had been warmed to 37° prior to use. The plates were incubated for a further 10 min at 37° and were then centrifuged at 500 *g* for 10 min. The supernatants were removed and assayed immediately for either lactoferrin or *N*-acetyl-β-D-glucosaminidase (NAG).

*Lactoferrin assay.* Lactoferrin was measured by an ELISA based on methods described by Hetherington *et al.* [24]. Ninety-six-well microtitre plates (Immunoplate 1; NUNC, Roskilde, Denmark) were coated with 100 μL of IgG fraction of rabbit anti-human lactoferrin (10 μg/mL; Cappel Laboratories, West Chester, PA) and incubated for 16 hr at 4°. The plates were washed three times with PBS containing 0.05% Tween 20. One hundred microliters of sample or purified human lactoferrin (Sigma) was added to the plates which were incubated for 1 hr at 37°. The plates were again washed and incubated with 100 μL of peroxidase conjugated IgG fraction of rabbit anti-human lactoferrin (10 μg/mL; Cappel Laboratories). After a further incubation of 1 hr at 37°, the plates were washed and 100 μL of peroxidase substrate (0.4 mg/mL *o*-phenylenediamine; 0.01% $H_2O_2$) was added. Color was allowed to develop for 10 min after which time the reaction was quenched by the addition of 150 μL of 8 N $H_2SO_4$ and the optical absorbance was read at 490 nm.

*NAG assay.* NAG was assayed using a micro-plate version of a method described by Woolen *et al.* [25]. A sample (75 μL) was added to a flat bottom 96-well tissue culture plate (Flow Laboratories, McLean, VA) together with an equal volume of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (8 mM) in 0.1 M citrate-phosphate buffer, pH 4.5 (0.1 M citric acid; 0.1 M disodium orthophosphate), and incubated at 37° for 1 hr. Color was developed by the addition of 150 μL of 0.2 M glycine buffer, pH 10.0 (0.2 M glycine; 0.2 M NaCl; 0.2 M NaOH), and optical density was read at 405 nm.

*Statistical analysis.* Comparisons between groups of data were made using analysis of variance followed

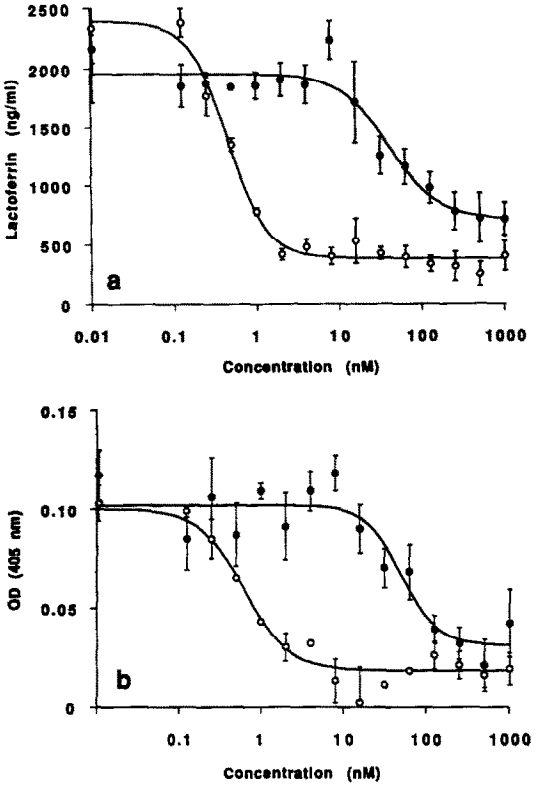


Fig. 1. Effects of CsA and FK-506 on A23187-induced PMNL degranulation. Degranulation of human peripheral blood PMNL was measured by (a) lactoferrin release and (b) *N*-acetyl-β-D-glucosaminidase (NAG) release. Cells were stimulated *in vitro* with the calcium ionophore A23187 ($10^{-6}$ M) in the presence of different concentrations of either CsA (●) or FK-506 (○). Each point is the mean of triplicate determinations within a single experiment with vertical bars representing SD. A best-fit curve through the data was drawn using a four-parameter fit program.

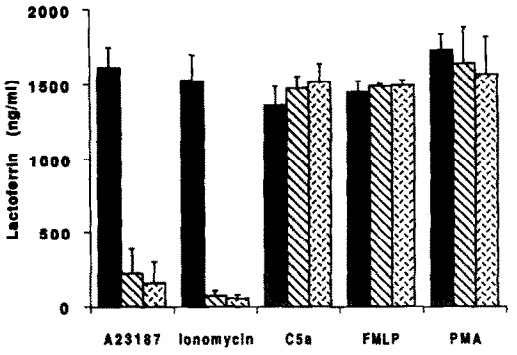


Fig. 2. Effects of CsA and FK-506 against different PMNL-activating stimuli. Degranulation of human PMNL, determined by the release of lactoferrin, was measured in response to A23187 ($10^{-6}$ M), ionomycin ($3 \times 10^{-6}$ M), C5a ($10^{-9}$ M), FMLP ($10^{-8}$ M) and PMA ($3 \times 10^{-9}$ M). PMNLs were preincubated with either HBSS (■), CsA (▨; 1 μM) or FK-506 (▨; 4 nM). Each column represents the mean of three separate experiments with vertical lines representing SD.

by Scheffe's multiple comparisons. $P < 0.05$ was considered to be significant.

## RESULTS

*Effects of CsA and FK-506 on A23187-induced PMNL degranulation.* Human PMNL degranulation in response to the calcium ionophore A23187 ($10^{-6}$ M) was assessed by the release of both NAG, a marker of azurophil granules, and lactoferrin, a marker of specific granules [26]. The incubation of PMNLs with either CsA or FK-506 produced a concentration-related inhibition of degranulation (Fig. 1, a and b). The concentrations of immunosuppressant required for 50% inhibition ($IC_{50}$) of either azurophil (50.8 and 0.63 nM for CsA and FK-506, respectively) or specific (41.0 and 0.46 nM) granule release were similar. In a series of experiments the mean $IC_{50}$ values for inhibition of specific granules were 33.7 ± 12.0 and 0.7 ± 0.09 nM (mean ± SEM; N = 7) for CsA and FK-506, respectively. It is apparent that some 50–100 times more CsA than FK-506 was required for inhibition of A23187-induced PMNL degranulation. This ratio is very similar to the ratio of CsA to FK-506 required for immunosuppressive activity [27, 28]. When $10^{-6}$ M A23187 was used to induce degranulation, FK-506 and CsA produced incomplete (70–75%) inhibition (Fig. 1, a and b), but at lower concentrations of A23187 total inhibition of degranulation was obtained (data not shown). When maximally effective concentrations of CsA (1 μM) and FK-506 (4 nM) were combined, the percent inhibition of degranulation in response to A23187 ($10^{-6}$ M) (69%) was no different from that obtained using either CsA (75%) or FK-506 alone (74%).

*Effects of CsA and FK-506 against different PMNL activating stimuli.* The immunosuppressive activities of both CsA and FK-506 *in vitro* [14, 15] and the inhibition of PMNL degranulation by CsA *in vitro* [17] are reported to be dependent on the nature of the activating signal. With respect to effects on lymphocytes the stimuli that are inhibited by CsA and FK-506 all produce a rise in intracellular calcium. In PMNLs the situation is less clear as stimuli which are reported to produce a rise in intracellular calcium such as zymosan-activated plasma [29] and FMLP [30] are not reported to be inhibited by CsA [18]. In Fig. 2 we demonstrate that both CsA and FK-506 inhibited only calcium ionophore (either A23187 or ionomycin) induced degranulation and not that induced by either receptor-mediated stimuli (C5a or FMLP) or through direct activation of protein kinase C (PMA). The inability of CsA and FK-506 to inhibit C5a- and FMLP-induced degranulation was also evident when sub-maximal concentrations of agonist were used to induce degranulation (data not shown). Thus, it would appear, at least for PMNLs, that a rise in intracellular calcium itself is not the sole determinant of whether CsA or FK-506 will have an inhibitory effect on cell activation. Rather, the inhibitory effect is restricted to stimuli which produce a translocation of calcium into the cell from the external medium. To test this hypothesis we used a

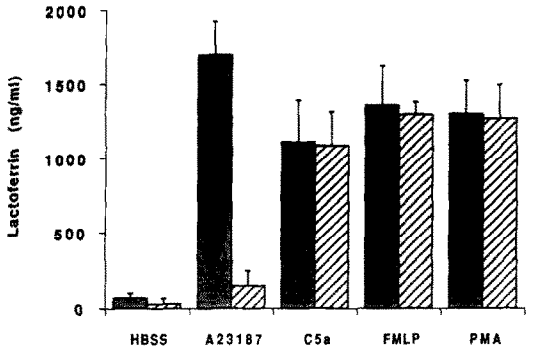


Fig. 3. Requirement of different PMNL-activating stimuli for extracellular calcium. Degranulation of human PMNL, determined by the release of lactoferrin, was measured in response to A23187 ($10^{-6}$ M), ionomycin ($3 \times 10^{-6}$ M), C5a ($10^{-9}$ M), FMLP ($10^{-8}$ M) and PMA ($3 \times 10^{-9}$ M). PMNLs were preincubated with either HBSS (▨) or EGTA (▨; 4 mM) for 5 min prior to addition of stimuli. Each column represents the mean of three separate experiments with vertical lines representing SD.

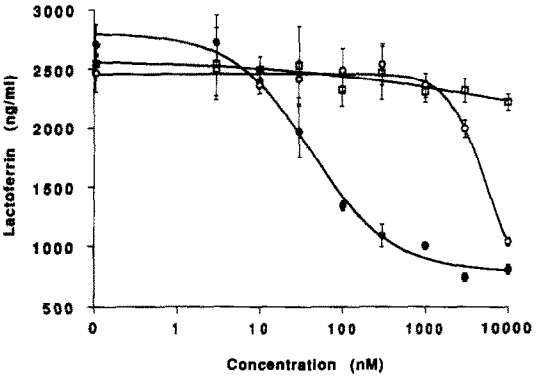


Fig. 4. Effects of CsA analogs on A23187-induced PMNL degranulation. Lactoferrin release from human peripheral blood PMNL was measured by ELISA following stimulation of cells with the calcium ionophore A23187 ($10^{-6}$ M) in the presence of different concentrations of either CsA (●), 6-methyl-alanine CsA (□) or 11-methyl-leucine CsA (○). Each point is the mean of triplicate determinations within a single experiment with vertical bars representing SD. A best-fit curve through the data was drawn using a four-parameter fit program.

protocol designed to remove extracellular calcium from the incubation medium but not to affect intracellular calcium stores. PMNLs were incubated with ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA, 4 mM) for 5 min prior to stimulation with agonists prepared in buffer containing 4 mM EGTA. Using this protocol (Fig. 3), degranulation of PMNL induced by A23187 in the presence of EGTA was inhibited substantially ($P < 0.05$), whereas C5a-, FMLP- or PMA-induced degranulation were unaffected ($P > 0.05$).

*Effects of rapamycin and non-immunosuppressive analogs of CsA on PMNL degranulation.* Because FK-506 and CsA inhibit T-cell activation and PMNL degranulation at similar concentrations, we wanted to address whether similar signal transduction pathways are being inhibited in both cell types. One approach to this question is to determine whether the inhibition of T-cell activation and PMNL degranulation occurs equally with different analogs of CsA and FK-506. We selected two analogs of CsA, 6-methyl-alanine CsA and 11-methyl-leucine CsA, which lack immunosuppressive activity. The two analogs differ in that 6-methyl-alanine CsA binds to cyclophilin, whereas 11-methyl-leucine CsA does not [31]. A representative experiment is shown in Fig. 4 demonstrating that 6-methyl-alanine CsA had no inhibitory activity at concentrations up to 10 μM. 11-Methyl-leucine CsA did produce inhibition but this effect was only seen at concentrations of 3 μM or greater and may represent a non-specific toxicity. In a series of three experiments, 11-methyl-leucine CsA at concentrations of 1 and 10 μM inhibited A23187-induced PMNL degranulation by 11.3 ± 6.8 and 42.4 ± 13.5%, respectively, whereas 6-methyl-alanine CsA inhibited degranulation by 6.7 ± 2.0 and 16.6 ± 8.0% at the same concentrations.

Rapamycin, a macrolide antibiotic with structural similarity to FK-506 [32], binds to the FK-506 binding protein, resulting in inhibition of its peptidylprolyl isomerase activity,* and has immunosuppressive activity [33]. However, the mechanism by which rapamycin inhibits murine T-cell proliferation is distinct from that of FK-506 or CsA [34]. For example, it does not inhibit interleukin-2 (IL-2) gene expression and does not show any of the selectivity of FK-506 and CsA for calcium-associated activation pathways in the lymphocyte. In addition, FK-506 and rapamycin could be shown to act as reciprocal antagonists in T-cell activation [35]. In the PMNL degranulation assay, rapamycin at concentrations up to 100 ng/mL did not inhibit A23187-induced PMNL degranulation (Fig. 5). However, when increasing concentrations of rapamycin were added to cells in the presence of a fixed inhibitory concentration of FK-506 (1 nM), rapamycin reversed the inhibition of degranulation.

*Effects of actinomycin D and cycloheximide on A23187 and PMA-induced PMNL degranulation.* The immunosuppressive activities of CsA and FK-506 are dependent ultimately on the inhibition of transcription of a number of lymphokine genes in lymphocytes. Because degranulation of neutrophils is so rapid, it is unlikely that transcriptional or translational events are involved in degranulation. However, to be certain that this was not the case, we examined the effects of actinomycin D and cycloheximide on A23187- and PMA-induced degranulation. Figure 6 demonstrates that preincubation of neutrophils with either actinomycin D or cycloheximide at concentrations of 1–10 μg/mL for 30 min had no effect ($P > 0.05$) on either A23187- or PMA-induced degranulation.

* Siekierka JJ, Harrison RK, Lin CS, Hung SHY and Sigal NH, Inhibition of FK-506 binding protein peptidyl-prolyl *cis-trans* isomerase activity is not directly related to immunosuppression. Manuscript submitted for publication.

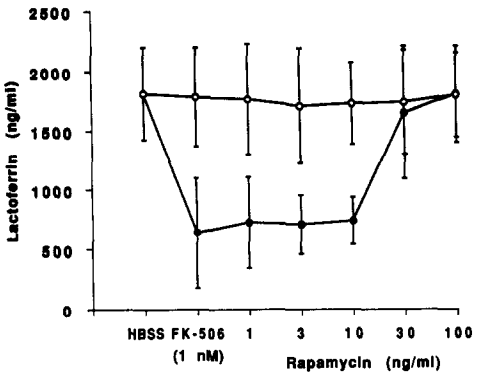


Fig. 5. Effect of rapamycin on A23187-induced PMNL degranulation. Lactoferrin release from human peripheral blood PMNL was measured by ELISA following stimulation of cells with the calcium ionophore A23187 ($10^{-6}$ M) in the presence of different concentrations of either rapamycin (○) or a fixed concentration of FK-506 (1 nM) plus different concentrations of rapamycin (●). Each point is the mean of three separate experiments with vertical bars representing SD.

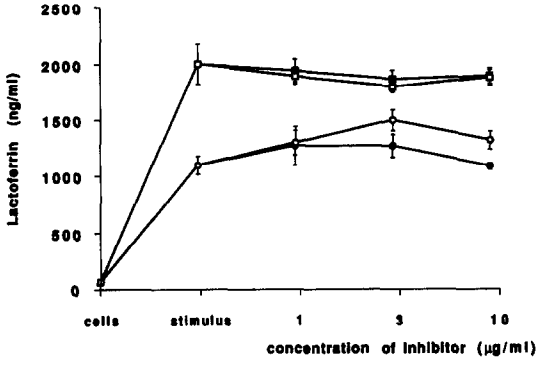


Fig. 6. Effects of actinomycin D and cycloheximide on A23187-induced PMNL degranulation. Lactoferrin release from human peripheral blood PMNLs was measured by ELISA following stimulation of cells with the calcium ionophore A23187 (○, ●; $10^{-6}$ M) of PMA (□, ■; $3 \times 10^{-9}$ M) in the presence of different concentrations of either actinomycin D (●, ■) or cycloheximide (○, □). Each point is the mean of triplicate determinations with vertical bars representing SD.

## DISCUSSION

In this paper we have demonstrated that both CsA and FK-506 inhibit calcium ionophore-induced PMNL degranulation at concentrations equivalent to those required for *in vitro* immunosuppressive activity. The inhibitory activities of CsA and FK-506 were restricted to calcium ionophore-induced degranulation, with responses to C5a, FMLP and PMA being unaffected by either CsA or FK-506. Analogs of CsA, which have been shown previously to lack immunosuppressive activity, were also ineffective as inhibitors of PMNL degradation. Rapamycin, a macrolide antibiotic structurally related to FK-506, was inactive against PMNL degranulation. Rapamycin does have immunosuppressive activity although its mechanism of action is clearly different from that of FK-506 [34]. Interestingly, rapamycin has been shown to antagonize some of the effects of FK-506 on T cells [35], and we also found that rapamycin antagonized the inhibitory effect of FK-506 on PMNL degranulation. These data support a hypothesis that FK-506 and CsA inhibit similar steps in a signal transduction pathway in both lymphocytes and PMNL.

A selective inhibitory effect of CsA for calcium ionophore-induced PMNL degranulation has been reported previously [17]. Our data not only confirm this previous finding but extend the observation to include the novel immunosuppressive agent FK-506. In addition, we have demonstrated that the inhibitory action of CsA is not restricted to the use of micromolar concentrations but that CsA and FK-506 inhibit ionophore-induced degranulation using concentrations of drug equivalent to those required for immunosuppression and which can be obtained clinically.

CsA and FK-506 do not inhibit T-cell proliferation induced by all stimuli [14, 15]. Rather, they inhibit a subset of stimuli, all of which produce an increase in intracellular calcium. The increase in intracellular calcium in T-cells, by stimuli which are sensitive to CsA or FK-506, is a result of mobilization of extracellular calcium [36]. Inhibition of PMNL degranulation is also restricted to a subset of stimuli, calcium ionophores which also require extracellular calcium for the observed rise in intracellular calcium. Thus, CsA and FK-506 may inhibit a process dependent upon the translocation of calcium into the cell rather than an increase in intracellular calcium itself. Alternatively, the requirement for extracellular calcium may distinguish a subset of calcium-associated pathways in which a downstream biochemical event is sensitive to CsA and FK-506. Although we did not examine the effects of CsA and FK-506 on intracellular calcium levels in PMNLs in response to calcium ionophore, it would appear that in other cell types CsA and FK-506 do not alter intracellular calcium [15, 21, 37]. The possibility that CsA and FK-506 act as chelators of extracellular calcium would explain some of the biological effects of these compounds. However, the uptake of $^{45}Ca^{2+}$ in rat basophilic leukemia cells induced by aggregation of IgE receptors is not inhibited by CsA [21], making this an unlikely explanation. Furthermore, if FK-506 were chelating calcium, then the ability of rapamycin to reverse the inhibitory effects of FK-506 would require that rapamycin physically interacts with FK-506, which has not been demonstrated previously.

There is clearly an abundance of evidence to suggest that CsA and FK-506 affect identical or closely related activation pathways in both PMNLs and T-cells. However, this does not imply an identical biochemical site of action for both CsA and FK-506, since discreet intracellular binding proteins for CsA [7] and for FK-506 [8] have been identified. Although the binding proteins both exhibit peptidyl-propyl isomerase activity [9–11], it is not clear whether the binding of CsA to cyclophilin or FK-506 to FKBP

is causally related to immunosuppression or in our experiments inhibition of PMNL degranulation. Our limited data with CsA analogs and with rapamycin would argue against a direct role for the binding proteins. 6-Methyl-alanine CsA binds to cyclophilin, whereas 11-methyl-leucine CsA shows only weak binding activity [31]. However, it was 11-methyl-leucine CsA which inhibited PMNL degranulation and only at concentrations of 3 $\mu$M or greater which may represent non-specific toxicity. In a study of a larger set of CsA analogs, Durette *et al.* [31] found a correlation between binding activity to cyclophilin and immunosuppressive activity. 6-Methyl-alanine CsA was an exception to this correlation as it bound but had no immunosuppressive activity. Similarly, rapamycin bound to FKBP* and did not inhibit PMNL degranulation, although it did antagonize the effects of FK-506. The investigation of a larger number of compounds and a greater understanding of the role of the binding proteins would be required in order to conclusively ascribe a role to the binding proteins in either the immunosuppressive or anti-degranulating activities of CsA and FK-506.

An important ramification for the observation of inhibitory effects of CsA and FK-506 in PMNL is that these signal transduction pathways may be operative in many other cell types, and their inhibition by immunosuppressive agents may be responsible for some of the toxic effects seen with immunosuppressants. A number of examples exist of CsA interacting with cells other than lymphocytes [38–43]. In many cases the concentrations of CsA used are well in excess of that required for immunosuppression. In contrast, the effects of CsA on cytolytic T-cell function [44] and the release of mediators from human basophils [20–22], human mast cells [19], rat mast cells [20] and rat basophilic leukemia cells [21] are seen at similar concentrations (approximately 100 ng/mL) to those reported here. The inhibition of release is seen when cells are stimulated with calcium ionophore or anti-IgE antiserum with lesser effects when specific antigen or concanavalin A is used. Mast cell degranulation in response to calcium ionophore and to anti-IgE is dependent on extracellular calcium [45] and produces a rise in intracellular calcium [46]. These data would be consistent with CsA affecting a similar signal transduction pathway to that described here in PMNLs.

Mediator release from basophils, mast cells and neutrophils induced by calcium ionophores is a rapid process unlikely to be dependent upon transcriptional or translational events. This contention is supported by the demonstration that actinomycin D and cycloheximide did not reduce A23187- or PMA-induced PMNL degranulation (Fig. 6). Therefore, it is unlikely that CsA and FK-506 inhibit PMNL degranulation by preventing gene expression. Since the data presented suggest that CsA and FK-506 affect similar activation pathways, we view it as unlikely that these immunosuppressants act directly at the transcriptional level in the lymphocyte. In particular, the notion that FK-506 and CsA act by inhibition of peptidyl-propyl isomerase catalyzed folding of lymphocyte-specific transcription factors may not be correct in light of the results in PMNL and other cell types. The current data are most consistent with the hypothesis that the drugs act at a biochemical step in a signal transduction pathway common to several different cell types but which ultimately subserves different intracellular functions.

We have presented evidence that both CsA and the more recently discovered immunosuppressant FK-506 can affect PMNL function at concentrations identical to those which produce immunosuppression *in vitro*. These data complement recent findings of an inhibitory effect of CsA on cytolytic T-cell function and mediator release from basophils and mast cells. The extent to which these effects on non-lymphoid cells may contribute to *in vivo* immunosuppression is unclear. The fact that other physiological stimuli of PMNL are not sensitive to either CsA or FK-506 would argue that inhibition of PMNL does not contribute to *in vivo* immunosuppression. However, the possibility that similar signal transduction pathways exist in both lymphoid and non-lymphoid cell types may be relevant to the toxic effects of these compounds.

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## REFERENCES

1. Calne RY, White DJG, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD and Rolles K, Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* **ii**: 1323–1327, 1978.
2. Powles RL, Barrett AJ, Clink H, Kay HEM, Sloane J and McElwain TJ, Cyclosporin A for the treatment of graft-versus-host disease in man. *Lancet* **ii**: 1327–1331, 1978.
3. Starzl TE, Fung J, Venkataramman R, Todo S, Demetris AJ and Jain A, FK 506 for liver, kidney and pancreas transplantation. *Lancet* **ii**: 1000–1004, 1989.
4. Tocci MJ, Matkovich DA, Collier KA, Kwok P, Dumont F, Lin S, DeGudicibus S, Siekierka JJ, Clin J and Hutchinson NI, The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. *J Immunol* **143**: 718–726, 1989.
5. Kronke M, Leonard WJ, Depper JM, Arya SK, Wong-Staal F, Gallo RC, Waldmann TA and Greene WC, Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc Natl Acad Sci USA* **81**: 5214–5218, 1984.
6. Elliott JF, Lin Y, Mizel SB, Bleackley RC, Harnish DG and Paetkau V, Induction of interleukin 2 messenger RNA inhibited by cyclosporin A. *Science* **226**: 1439–1441, 1984.
7. Handschumacher RE, Harding MW, Rice J and Drugge RJ, Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* **226**: 544–547, 1984.
8. Siekierka JJ, Staruch MJ, Hung SHY and Sigal NH, FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. *J Immunol* **143**: 1580–1583, 1989.
9. Siekierka JJ, Hung SHY, Poe M, Lin CS and Sigal NH, A cytosolic binding protein for the

* Siekierka JJ, Harrison RK, Lin CS, Hung SHY and Sigal NH, Inhibition of FK-506 binding protein peptidyl-prolyl *cis-trans* isomerase activity is not directly related to immunosuppression. Manuscript submitted for publication.

immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**: 755–757, 1989.
10. Takahashi N, Hayano T and Suzuki M, Peptidyl-prolyn *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**: 473–475, 1989.
11. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T and Schmid FX, Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**: 476–478, 1989.
12. Siekierka JJ, Wiederrecht G, Greulich H, Boulton D, Hung SHY, Cryan J and Sigal NH, The cytosolic binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptidyl-prolyl *cis-trans* isomerase. *J Biol Chem* **265**: 21011–21015, 1990.
13. Koletsky AJ, Harding MW and Handschumacher RE, Cyclophilin: Distribution and variant properties in normal and neoplastic tissues. *J Immunol* **137**: 1054–1059, 1986.
14. June CH, Ledbetter JA, Gillespie MM, Lindsten T and Thompson CB, T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol Cell Biol* **7**: 4472–4481, 1987.
15. Lin CS, Boltz RC, Siekierka JJ and Sigal NH, FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human lymphocytes. *Cell Immunol* **133**: 269–284, 1990.
16. Janco RL and English D, Cyclosporine and human neutrophil function. *Transplantation* **35**: 501–503, 1983.
17. Kharazmi A, Svenson M, Nielsen H and Birgens HS, Effect of cyclosporin A on human neutrophil and monocyte function. *Scand J Immunol* **21**: 585–591, 1985.
18. Weinbaum DL, Kaplan SS, Zdziarski U, Rinaldo CR Jr and Schroeder KK, Human polymorphonuclear leukocyte interaction with cyclosporine A. *Infect Immun* **43**: 791–794, 1984.
19. Marone G, Triggiani M, Cirillo R, Giacummo A, Siri L and Condorelli M, Cyclosporin A inhibits the release of histamine and peptide leukotriene C4 from human lung mast cells. *Ric Clin Lab* **18**: 53–59, 1988.
20. Pedersen C, Permin H, Stahl Skov P, Norn S, Svenson M, Mosbech H and Bendtazen K, Inhibitory effect of cyclosporin A on histamine release from human leukocytes and rat mast cells. *Allergy* **40**: 103–107, 1985.
21. Hultsch T, Rodriquez JL, Kaliner MA and Hohman RJ, Cyclosporin A inhibits degranulation of rat basophilic leukemia cells and human basophils: Inhibition of mediator release without affecting PI hydrolysis or $Ca^{2+}$ fluxes. *J Immunol* **144**: 2659–2664, 1990.
22. Cirillo R, Triggiani M, Siri L, Ciccarelli A, Pettit GP, Condorelli M and Marone G, Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. *J Immunol* **144**: 3891–3897, 1990.
23. Sehgal SN, Baker H and Vezina C, Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J Antibiot (Tokyo)* **28**: 727–732, 1975.
24. Hetherington SV, Spitznagel JK and Quie PG, An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin. *J Immunol Methods* **65**: 183–190, 1983.
25. Woolen JW, Heyworth R and Walker PG, Studies on glucosaminidase. 3. Testicular *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase. *Biochem J* **78**: 111–116, 1961.
26. Baggiolini M and Dewald B, The neutrophil. *Int Arch Allergy Appl Immunol* **76**: 13–20, 1985.
27. Sawada S, Suzuki G, Kawase Y and Takaku F, Novel immunosuppressive agent, FK-506. *In vitro* effects on the cloned T cell activation. *J Immunol* **139**: 1797–1803, 1987.
28. Kino T, Hatanaka H, Miyata S, Inamura N, Nishiyama M, Yajima T, Goto T, Okuhara M, Kohsaka M, Aoki H and Ochiai T, FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. *J Antibiot (Tokyo)* **40**: 1256–1265, 1987.
29. Smith RJ and Ignarro LJ, Bioregulation of lysosomal enzyme secretion from human neutrophils: Roles of guanosine 3′:5′-monophosphate and calcium in stimulus–secretion coupling. *Proc Natl Acad Sci USA* **72**: 108–112, 1975.
30. Naccache PH, Showell HJ, Becker EL and Sha'afi RI, Transport of sodium, potassium and calcium across rabbit polymorphonuclear leukocyte membranes. Effect of chemotactic factor. *J Cell Biol* **73**: 428–444, 1977.
31. Durette PL, Boger J, Dumont F, Firestone R, Frankshun RA, Koprak SL, Lin CS, Melino MR, Pessolano AA, Pisano J, Schmidt JA, Sigal NH, Staruch MJ and Witzel BE, A study of the correlation between cyclophilin binding and *in vitro* immunosuppressive activity of cyclosporin A and analogues. *Transplant Proc* **20**: 51–57, 1988.
32. Findlay JA and Radics L, On the chemistry and high field nuclear magnetic resonance spectroscopy of rapamycin. *Can J Chem* **28**: 579–590, 1980.
33. Martel RR, Klicius J and Galet S, Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can J Physiol Pharmacol* **55**: 48–51, 1977.
34. Dumont FJ, Staruch MJ, Koprak SL, Melino MR and Sigal NH, Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J Immunol* **144**: 251–258, 1990.
35. Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA and Sigal NH, The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T-cells. *J Immunol* **144**: 1418–1424, 1990.
36. Gardner AP, Calcium and T-cell activation. *Cell* **59**: 15–20, 1989.
37. Metcalfe S, Cyclosporin does not prevent cytoplasmic calcium changes associated with lymphocyte activation. *Transplantation* **38**: 161–164, 1984.
38. Kukongviriyapan V and Stacey NH, Inhibition of taurocholate transport by cyclosporin A in cultured rat hepatocytes. *J Pharmacol Exp Ther* **247**: 685–689, 1988.
39. Inselmann G, Blank M and Baumann K, Cyclosporin A induced lipid peroxidation in microsomes and effect on active and passive glucose transport by brush border membrane vesicles of rat kidney. *Res Commun Chem Pathol Pharmacol* **62**: 207–220, 1988.
40. Zoja C, Furci L, Ghilardi F, Zilio P, Benigni A and Remuzzi G, Cyclosporin-induced endothelial cell injury. *Lab Invest* **55**: 455–462, 1986.
41. Pfeilschifter J, Cyclosporin A augments vasoconstrictor induced rise in intracellular free calcium in rat renal mesangial cells. *Biochem Pharmacol* **37**: 4205–4210, 1988.
42. Fan T-PD and Lewis JP, Mechanism of cyclosporin A-induced inhibition of prostacyclin synthesis by macrophages. *Prostaglandins* **30**: 735–747, 1985.
43. Boelsterli UA, Bouis P, Brouillard J-F and Donatsch P, *In vitro* toxicity assessment of cyclosporin A and its analogs in a primary rat hepatocyte culture model. *Toxicol Appl Pharmacol* **96**: 212–221, 1988.
44. Trenn G, Taffs R, Hohman RJ, Kincaid R, Shevach EM and Sitkovsky M, Biochemical characterization of

the inhibitory effect of CsA on cytolytic T lymphocyte effector functions. *J Immunol* **142**: 3796–3802, 1989.

45. Mongar JL and Schild HO, The effect of calcium and pH on the anaphylactic reaction. *J Physiol (Lond)* **140**: 272–284, 1958.
46. Ishizaka T, Foreman JC, Sterk AR and Ishizaka K, Induction of calcium flux across the rat mast cell membrane by bridging IgE receptors. *Proc Natl Acad Sci USA* **76**: 5858–5862, 1979.