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Immunology

Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A

(immunophilins/immunosuppression)

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ABSTRACT **The immunosuppressive agents cyclosporin A (CsA) and FK 506 bind to distinct families of intracellular proteins (immunophilins) termed cyclophilins and FK 506-binding proteins (FKBPs). Recently, it has been shown that, *in vitro*, the complexes of CsA–cyclophilin and FK 506–FKBP-12 bind to and inhibit the activity of calcineurin, a calcium-dependent serine/threonine phosphatase. We have investigated the effects of drug treatment on phosphatase activity in T lymphocytes. Calcineurin is expressed in T cells, and its activity can be measured in cell lysates. Both CsA and FK 506 specifically inhibit cellular calcineurin at drug concentrations that inhibit interleukin 2 production in activated T cells. Rapamycin, which binds to FKBPs but exhibits different biological activities than FK 506, has no effect on calcineurin activity. Furthermore, excess concentrations of rapamycin prevent the effects of FK 506, apparently by displacing FK 506 from FKBPs. These results show that calcineurin is a target of drug–immunophilin complexes *in vivo* and establish a physiological role for calcineurin in T-cell activation.**

The immunosuppressants cyclosporin A (CsA), FK 506, and rapamycin have proven to be valuable probes for studying T-cell signal transduction (1, 2). While CsA and FK 506 bind to distinct cellular receptors, these agents exhibit essentially identical effects on T-cell activation: both inhibit Ca^{2+}-dependent activation pathways that lead to transcription of lymphokine genes (1–6). Rapamycin, like FK 506, binds to FK 506-binding proteins (FKBPs), but inhibits T-cell activation by interfering with distinct Ca^{2+}-independent signaling pathways (7–9). Rapamycin reverses the action of FK 506, apparently by competitive binding to FKBPs (1, 2, 8, 9). These and other findings have led to the model that an immunosuppressant bound to its cellular receptor (immunophilin) forms an inhibitory complex that interferes with signal transduction (1, 2, 9–11). The complexes of CsA–cyclophilin and FK 506–FKBP are postulated to affect the same signaling component, while rapamycin–FKBP affects a distinct component.

Calcineurin, also known as phosphatase 2B, is a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase consisting of two subunits with predicted molecular masses of 59 kDa and 19 kDa (12). It is expressed ubiquitously in eukaryotic cells, including yeast (13). In mammals, calcineurin is most abundant in brain (12) but also has been detected in T cells (14, 15). Recently, it was reported that cyclophilin or FKBP-12 affinity matrices, in the presence of CsA or FK 506, respectively, could bind calcineurin from calf brain and thymus extracts (16, 17). Both chains of calcineurin were retained by the affinity matrices, and binding was Ca^{2+}-dependent. *In vitro*, CsA and FK 506 potently inhibited bovine brain calcineurin enzyme activity in the presence of the appropriate immunophilin. These *in vitro* studies suggested that CsA and FK 506 complexed to cellular immunophilins might inhibit calcineurin phosphatase activity in T cells and that calcineurin may play a critical role in Ca^{2+}-dependent T-cell activation. Therefore, we investigated whether calcineurin is a target of inhibition by CsA and FK 506 in T cells.

MATERIALS AND METHODS

Cells. Jurkat cells (clone J77), a gift of K. Smith (Dartmouth Medical School, Dartmouth, NH), were cultured in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 100 units of penicillin per ml, 100 μg of streptomycin per ml, 10 mM Hepes, 2 mM L-glutamine, and 25 μM 2-mercaptoethanol (termed "complete medium") at 37°C in humidified air containing 5% CO_2. PC12 cells were obtained from K. Wood (Dana–Farber Cancer Institute) and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FCS, 5% heat-inactivated horse serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine at 37°C in humified air containing 10% CO_2.

Immunoblotting (Western) Analysis. Cells (10^7) were lysed for 5 min on ice in 100 μl of buffer containing 0.5% Triton X-100; 50 mM Tris (pH 8); 150 mM NaCl; and 50 μg of phenylmethylsulfonyl fluoride, 50 μg of soybean trypsin inhibitor, 5 μg of leupeptin, and 5 μg of aprotinin per ml. Lysates were clarified by centrifugation at 4°C for 2 min at 12,000 × *g*. Proteins concentrations in the lysates were determined by using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. Proteins were subjected to SDS/PAGE with 12% polyacrylamide gels and electroblotted onto nitrocellulose. Filters were blocked for 1 hr in Tris-buffered saline (TBS) (1× TBS = 10 mM Tris, pH 8/150 mM NaCl) containing 5% Carnation nonfat dry milk and 0.02% NaN_3, were rinsed three times with TBS containing 0.05% Tween-20 (TBST), and were incubated for 2 hr with rabbit anti-bovine calcineurin antiserum (immunoglobulin fraction) diluted 1:1000 in TBST. After three washes in TBST, filters were incubated for 90 min with 5 μCi (185 Bq) of ^{125}I-labeled protein A (New England Nuclear) in TBST. Filters were then washed twice with TBS, twice with TBST, and twice with TBS before air-drying and exposure to Kodak X-Omat film.

Phosphatase Substrate. The peptide Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-

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Abbreviations: CsA, cyclosporin A; FKBP, FK 506-binding protein; IL-2, interleukin 2; PMA, phorbol 12-myristate 13-acetate.

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Glu, corresponding to a sequence in the RII subunit of cAMP-dependent kinase (18), was synthesized and sequenced by standard procedures (Molecular Biology Core Facility, Dana–Farber Cancer Institute). Phosphorylation of the serine residue with [γ-^{32}P]ATP was performed essentially as described (19) with the catalytic subunit of cAMP-dependent protein kinase (Sigma). The specific activity of fresh preparations was ≈50–75 μCi/μmol of peptide.

Inhibitory Peptide. Two peptides overlapping the autoinhibitory domain of calcineurin A identified by limited proteolysis (20) were synthesized and HPLC-purified (Peptide Technologies, Washington). Peptide 412 corresponds to residues 466–490 of the calcineurin A α chain (Ile-Thr-Ser-Phe-Glu-Glu-Ala-Lys-Gly-Leu-Asp-Arg-Ile-Asn-Glu-Arg-Met-Pro-Pro-Arg-Arg-Asp-Ala-Met-Pro). It was shown to inhibit specifically the phosphatase activity of calmodulin-activated or protease-activated calcineurin towards myosin light chains or *p*-nitrophenyl phosphate with a K_i value of 20 μM (21). Peptide 413 (Gly-Phe-Ser-Pro-Pro-His-Arg-Ile-Thr-Ser-Phe-Glu-Glu-Ala-Lys-Gly), which does not inhibit calcineurin at concentrations up to 50 μM (C.B.K., unpublished data), overlaps partially with peptide 412 and spans residues 469–484 of the calcineurin A β chain. Prior to assays, the peptides were subjected to gel filtration on Sephadex G-10 in 50% acetic acid to remove any small molecular weight contaminants. After lyophilization, the peptides were solubilized in water at concentrations up to 3–4 mM. Phenylalanine absorbance at 258 nm was used to determine the peptide concentrations; molar extinction coefficients of 192 for peptide 412 and 384 for peptide 413 were used. The sequences of the peptides were verified by protein sequencing on a model 477A Applied Biosystems protein sequencer.

Cell Treatment and Lysis. Immunosuppressive agents were dissolved in ethanol at concentrations 1000-fold more than the concentration desired for cell treatments. Cells (10^6) were suspended in 1 ml of complete medium in microcentrifuge tubes; 1 μl of ethanol or of the ethanolic solution of FK 506, CsA, or rapamycin was added, and the cells were incubated at 37°C for 1 hr. Cells were washed twice with 1 ml of phosphate-buffered saline (PBS) on ice and lysed in 50 μl of hypotonic buffer containing 50 mM Tris (pH 7.5); 0.1 mM EGTA; 1 mM EDTA; 0.5 mM dithiothreitol; and 50 μg of phenylmethylsulfonyl fluoride, 50 μg of soybean trypsin inhibitor, 5 μg of leupeptin, and 5 μg of aprotinin per ml. Lysates were subjected to three cycles of freezing in liquid nitrogen followed by thawing at 30°C and then were centrifuged at 4°C for 10 min at 12,000 × *g*.

Phosphatase Assay. Purified bovine brain calcineurin and calmodulin were purchased from Sigma. Reaction mixtures with purified enzyme contained 100 nM calcineurin, 100 nM calmodulin, and 5 μM ^{32}P-labeled phosphopeptide, in 60 μl (total volume) of assay buffer containing 20 mM Tris (pH 8), 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, and either 0.1 mM $CaCl_2$ or 5 mM EGTA. Reaction mixtures with cell lysates contained 20 μl of undiluted lysate, 5 μM ^{32}P-labeled phosphopeptide, and 40 μl of assay buffer. Where indicated, reaction mixtures contained 50 μM peptide 412 or 413 and/or 500 nM okadaic acid, a specific inhibitor of phosphatases 1 and 2A (22, 23); 500 nM okadaic acid is sufficient for inhibition of Ca^{2+}-independent phosphatases, whereas higher concentrations partially inhibit Ca^{2+}-dependent activity as well (unpublished observations). After 15 min at 30°C, reactions were terminated by the addition of 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5% trichloroacetic acid. Free inorganic phosphate was isolated by Dowex cation-exchange chromatography and quantitated by scintillation counting as described (24). Assays were performed in duplicate, and the cpm measured in blank assays lacking enzyme were subtracted from the total cpm. With freshly labeled substrate, a 60-μl assay contained ≈50,000 input cpm; after Dowex chromatography, typical blank and maximum values were 1700 cpm and 22,000 cpm, respectively. Variation between duplicates was <10%. The number of picomoles of phosphate released was calculated by using the specific activity of the substrate measured on the day of the assay. Specific activity was determined by measuring the cpm in 20 μl of 15 μM (300 pmol) ^{32}P-labeled phosphopeptide.

Interleukin 2 (IL-2) Assay. Jurkat cells were cultured in complete medium at 10^6 cells per ml in 96-well flat-bottom plates. Cells were stimulated with OKT3 monoclonal antibody (1:4000 dilution of ascites) and 2 ng of phorbol 12-myristate 13-acetate (PMA) per ml for 24 hr in the presence or absence of FK 506 or CsA. IL-2 production was quantitated by measuring the ability of serial dilutions of cell supernatants to support the proliferation of the IL-2-dependent cell line CTLL-20 as described (25, 26). One unit is defined as the amount of recombinant human IL-2 required to induce half-maximal proliferation of the CTLL-20 cells. FK 506 and CsA added directly to CTLL-20 cells do not inhibit IL-2-dependent proliferation (26).

RESULTS

Analysis of Calcineurin Expression. Calcineurin expression was assessed in the Jurkat human leukemia T-cell line J77. These cells produce IL-2 when stimulated with a combination of PMA and OKT3, a monoclonal antibody directed against the T-cell receptor–CD3 complex (26). Immunoblotting experiments with an antiserum that recognizes both the A and B chains of calcineurin confirmed that both subunits are expressed in Jurkat T cells (Fig. 1). Calcineurin B, which consistently migrates at 16 kDa in SDS/polyacrylamide gels, is detected along with a predominant calcineurin A band migrating at 61 kDa. The band at 28 kDa is probably a proteolytic fragment of calcineurin A generated during preparation of cell lysates (C.B.K., unpublished observations). The immunoreactive band migrating above 61 kDa may represent another calcineurin isoform, since it is also observed in the purified calcineurin preparation and in PC12 cells, which reportedly express several molecular isoforms of calcineurin A (27).

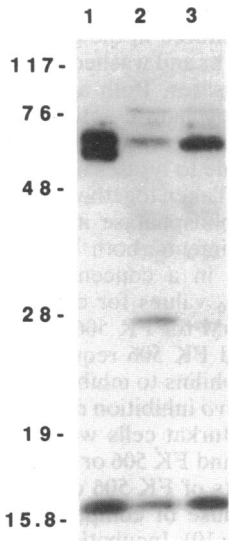

FIG. 1. Immunoblot analysis of calcineurin expression. Proteins were resolved by SDS/PAGE (12% polyacrylamide gel), transferred to nitrocellulose, and probed with an antiserum that recognizes both the A and B chains of calcineurin. Lanes: 1, purified bovine brain calcineurin (200 ng); 2, Jurkat cell lysate (100 μg of protein); 3, PC12 cell lysate (100 μg of protein). Size markers are indicated on the left.

Calcineurin Phosphatase Activity in Jurkat T Cells. Given that calcineurin was readily detectable by Western analysis, we attempted to measure calcineurin enzyme activity in J77 cell lysates. A peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (18) was synthesized and phosphorylated *in vitro* as described in *Materials and Methods*. Dephosphorylation of this substrate by purified calcineurin is Ca^{2+}-dependent and resistant to okadaic acid (Fig. 2 *Top* and ref. 20), a potent inhibitor of phosphatases 1 and 2A (22, 23). Lysates of Jurkat cells also contained enzymatic activity able to dephosphorylate this substrate in the presence of Ca^{2+} (Fig. 2 *Middle*). When 500 nM okadaic acid was included in the assay to inhibit the activity of phosphatases 1 and 2A, nearly all of the remaining phosphatase activity was Ca^{2+}-dependent, and could be eliminated by substituting 5 mM EGTA for Ca^{2+} (Fig. 2 *Middle*). In contrast, the okadaic acid-sensitive component was resistant to EGTA (Fig. 2 *Middle*), which is consistent with the reported Ca^{2+}-independence of phosphatases 1 and 2A (12). Taken together, these results indicate that Ca^{2+}-dependent phosphatase activity can be measured in Jurkat cell lysates.

Calcineurin is the only known okadaic acid-insensitive, Ca^{2+}-dependent phosphatase. To further verify that calcineurin was responsible for the phosphatase activity observed in the presence of Ca^{2+} and okadaic acid, a peptide inhibitor of calcineurin was included in the assay. The peptide corresponds to a sequence in the autoinhibitory domain of the calcineurin A subunit (21). Although the peptide is a relatively weak inhibitor *in vitro* (21), its action is specific because it does not affect Ca^{2+}-independent, okadaic acid-sensitive phosphatases (21). At 50 μM, this peptide inhibited the activity of purified calcineurin by about 60%, whereas a control peptide had no effect (Fig. 2 *Top*). When added to Jurkat lysates, the inhibitory peptide inhibited the Ca^{2+}-dependent, okadaic acid-insensitive component by approximately 50% (Fig. 2 *Middle*). These findings support the conclusion that the cellular phosphatase activity measured in the presence of Ca^{2+} and okadaic acid is attributable to calcineurin.

Inhibition of Calcineurin Activity by Immunosuppressive Drugs. Both CsA and FK 506 potently inhibit IL-2 production by Jurkat cells stimulated with OKT3 plus PMA (26). To assess whether drug treatment inhibits calcineurin activity, Jurkat cells were cultured in the presence of 10 nM FK 506 or 100 nM CsA for 1 hr and washed, and phosphatase activity was measured in lysates. Both agents effectively inhibited Ca^{2+}-dependent phosphatase activity, while Ca^{2+}-independent activity was unaffected (Fig. 2 *Bottom*). Addition of 50 μM inhibitory peptide to lysates of drug-treated cells did not augment inhibition. Taken together, these results suggest that the drug-sensitive phosphatase in Jurkat is calcineurin. In drug titration experiments, both FK 506 and CsA inhibited calcineurin activity in a concentration-dependent fashion (Fig. 3 *Upper*). IC_{50} values for calcineurin inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA.

In vitro, CsA and FK 506 require complexation to their respective immunophilins to inhibit calcineurin activity (16). To determine if *in vivo* inhibition requires drug complexation to immunophilins, Jurkat cells were treated with combinations of rapamycin and FK 506 or CsA. Rapamycin prevents the inhibitory effects of FK 506 on Ca^{2+}-dependent activation, probably because of competition for FKBP receptor binding sites (1, 2, 8–10). Incubation with rapamycin alone at concentrations up to 1 μM had no effect on cellular calcineurin activity (Fig. 3 *Upper*). However, at 1 μM, rapamycin prevented FK 506-mediated inhibition of calcineurin activity (Fig. 3 *Lower*). An excess of rapamycin is required for FK 506 antagonism, possibly because the high concentration of FKBPs in T cells acts to buffer the added drug (1, 2, 8–10).

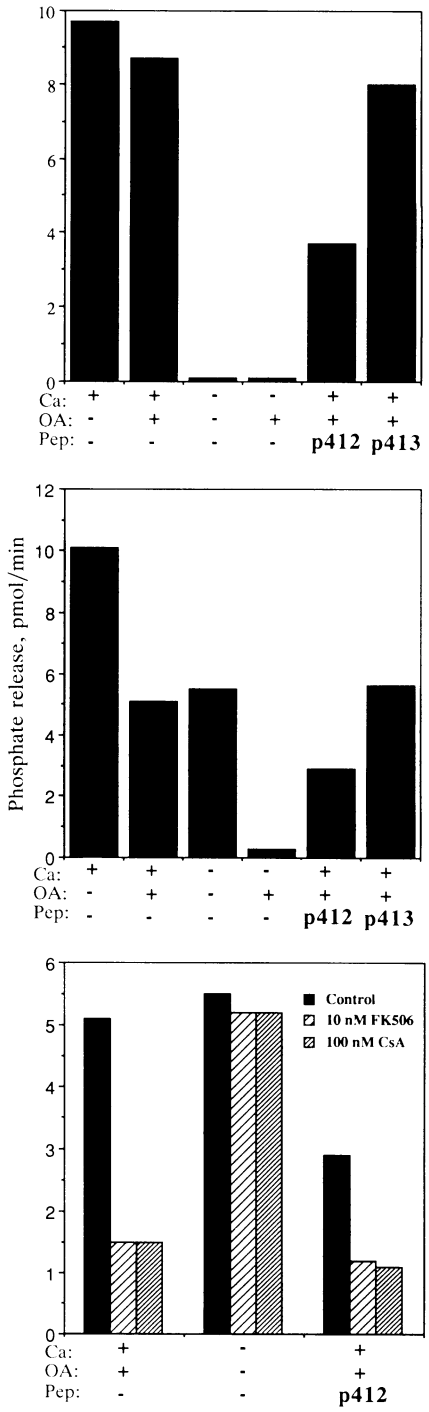

FIG. 2. Dephosphorylation of a synthetic peptide substrate by purified calcineurin and cell lysates. Phosphatase assays were performed in the presence or absence of Ca^{2+}, okadaic acid (OA), and peptides (Pep) as indicated. (*Top*) Purified calcineurin. (*Middle*) Lysates of Jurkat T cells. (*Bottom*) Lysates from Jurkat cells cultured with immunosuppressive agents. Phosphatase activity is expressed as picomoles of phosphate released per minute. Protein concentrations in lysates of drug-treated and control cells were equivalent.

Rapamycin antagonism was specific because the drug failed to prevent the effects of CsA (Fig. 3 *Lower*). These results indicate that inhibition by FK 506 is due to FK 506–FKBP complexes formed during drug treatment. Since rapamycin does not bind to cyclophilins, inhibition by CsA–cyclophilin complexes is unaffected by rapamycin.

Correlation of Calcineurin Activity and IL-2 Production. The finding that immunosuppressive agents inhibit calcineurin activity in T cells suggests that this phosphatase is

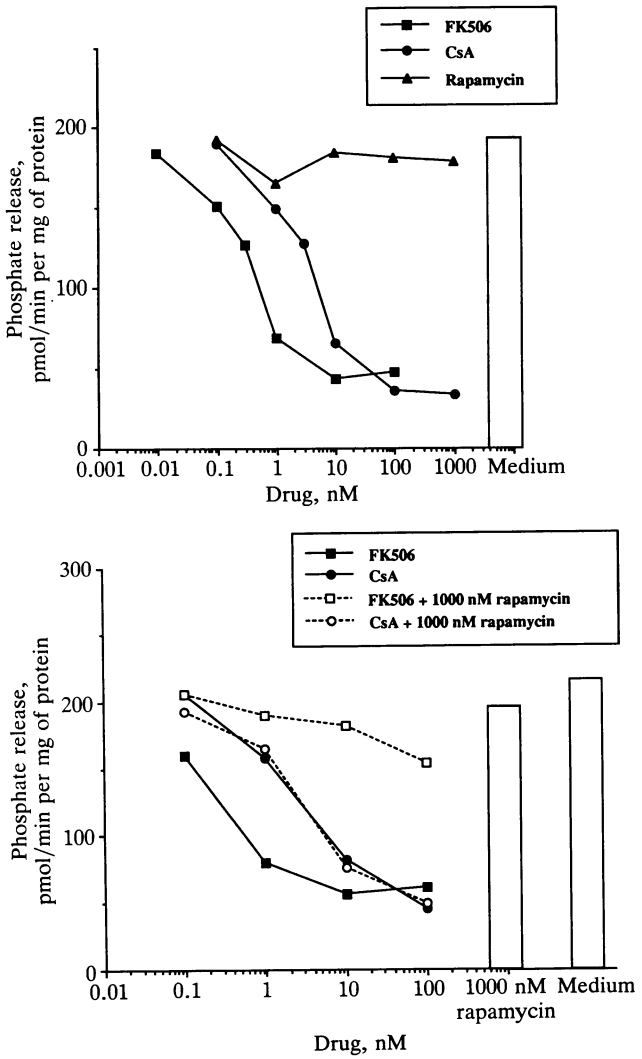

FIG. 3. Drug titration and rapamycin reversibility. Phosphatase activity is expressed as pmoles phosphate released per minute per mg of cell protein. (*Upper*) Concentration dependence of FK 506 and CsA inhibition of calcineurin and lack of effect by rapamycin. (*Lower*) Rapamycin antagonism of FK 506-mediated inhibition.

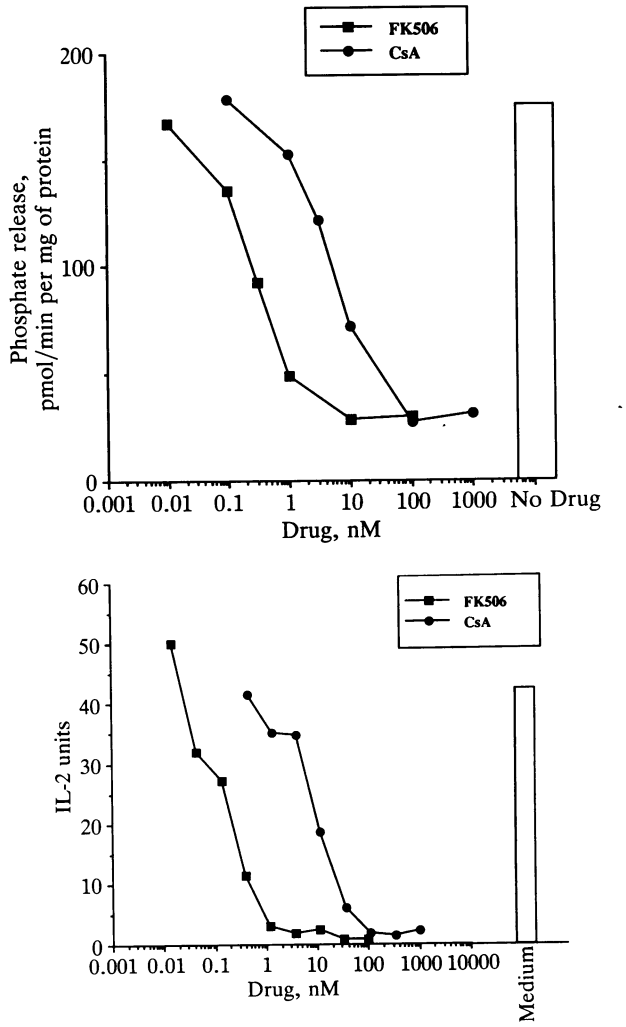

FIG. 4. Comparison of calcineurin activity and IL-2 production. Calcineurin activity (*Upper*) and IL-2 production (*Lower*) in cells stimulated with OKT3 plus PMA in the presence of immunosuppressive drugs. IL-2 release was quantitated by using the IL-2-dependent cell line CTLL-20.

important for signal transduction during T-cell activation. This hypothesis predicts that the level of calcineurin activity should correlate with the level of T-cell activation. Therefore, we used FK 506 and CsA as specific inhibitors to vary the amount of calcineurin activity in Jurkat cells stimulated with OKT3 plus PMA. In our assay system, the levels of calcineurin activity measured in untreated and stimulated cells are similar. FK 506 and CsA inhibited calcineurin in stimulated cells with IC_{50} values of 0.4 nM and 7 nM, respectively (Fig. 4 *Upper*). In parallel experiments, Jurkat cells were incubated with OKT3 and PMA and various concentrations of CsA or FK 506 for 24 hr, and IL-2 production was measured. IC_{50} values for inhibition of IL-2 production were approximately 0.2 nM for FK 506 and 10 nM for CsA (Fig. 4 *Lower*). The strong correlation in drug sensitivity suggests a direct relationship between calcineurin phosphatase activity and T-cell activation.

DISCUSSION

To analyze the effects of immunosuppressive agents on calcineurin activity in T cells, a biochemical assay to measure phosphatase activity in cell lysates has been developed. In the presence of okadaic acid, dephosphorylation of a synthetic peptide substrate is both Ca^{2+}-dependent and sensitive to inhibition by a specific inhibitor of calcineurin (Fig. 2). When Ca^{2+} and okadaic acid are omitted, dephosphorylation mediated by other cellular phosphatases can be measured. It should be noted that, in this assay system, no difference in calcineurin activity is detected between unstimulated cells and cells activated with OKT3 plus PMA (compare Figs. 3 *Upper* and 4 *Upper*). Presumably, since sufficient Ca^{2+} is present in the phosphatase assay buffer (0.1 mM), free calcineurin in cell extracts becomes activated regardless of the stimulus applied before cell lysis. This observation also suggests that any modifications of calcineurin that might accompany activation (e.g., phosphorylation) do not affect its enzyme activity in the presence of 0.1 mM Ca^{2+}.

Calcineurin activity is potently inhibited when Jurkat T cells are treated with FK 506 or CsA (Figs. 2 and 3). Inhibition appears to be specific for calcineurin, since the activity of okadaic acid-sensitive phosphatases (i.e., phosphatases 1 and 2A) is not affected by either agent. Rapamycin prevents the inhibition of calcineurin mediated by FK 506 but not CsA (Fig. 3 *Lower*). The latter finding is consistent with the model in which complexes formed by FK 506–FKBP and CsA–cyclophilin are responsible for inhibition of the cellular target (1, 2, 9, 10).

Drug titration experiments demonstrate that the inhibition of cellular calcineurin closely parallels the inhibition of T-cell activation as assessed by IL-2 production (Fig. 4). These findings suggest that calcineurin activity is essential for Ca^{2+}-dependent T-cell activation. The strict Ca^{2+}-dependence of its phosphatase activity makes calcineurin an attractive candidate for such a pathway. Identification of the cellular target of drug–immunophilin complexes allows innovative approaches to the development of immunosuppressive agents. It may be possible to bypass the requirement for immunophilin binding by designing direct inhibitors of calcineurin. In the meantime, since FKBPs and cyclophilins are widely expressed (1), FK 506 or CsA can be used as specific inhibitors to study the function of calcineurin in many cell types.

If calcineurin is a critical enzyme in Ca^{2+}-dependent activation pathways, it is interesting that the biological effects of FK 506 and CsA are relatively specific for T cells. A potential explanation is that these agents may preferentially affect cells in which immunophilins are in excess of calcineurin. While Jurkat cells express high levels of immunophilins (1), quantitation of protein levels by Western blot indicates that calcineurin is expressed at 25-fold lower levels in Jurkat cells than in brain tissue (unpublished data). Perhaps a similar pattern of calcineurin and immunophilin expression occurs in renal cells, resulting in the nephrotoxicity commonly observed in patients treated with FK 506 or CsA (28).

To understand the role of calcineurin in T-cell activation, it will be important to identify its substrates. It has been proposed that calcineurin dephosphorylates the cytoplasmic subunit of the transcription factor NF-AT, allowing it to translocate to the nucleus where it is involved in transcriptional activation of lymphokine genes (29, 30). In this regard, nuclear translocation of a transcription factor in yeast has been reported to be associated with a decrease in serine/threonine phosphorylation (31). The ability to measure directly calcineurin activity in cell lysates should facilitate the further analysis of this phosphatase and its potential physiological substrates.

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