Cyclosporine- and FK506-Induced Sympathetic Activation Correlates With Calcineurin-Mediated Inhibition of T-Cell Signaling


Cyclosporine A (CsA) has greatly improved long-term survival after organ transplantation and the treatment of autoimmune diseases, but it also causes significant hypertension in large numbers of patients. Although CsA-induced hypertension seems to be caused in part by sympathetic overactivity with neurogenic vasoconstriction, the underlying mechanism by which CsA activates the sympathetic nervous system is unknown.

The effects of CsA and the investigational immunosuppressive drugs FK506 and rapamycin on T-cell signaling are dependent on their interactions with two different cytoplasmic receptors, termed immunophilins: cyclophilin, which binds CsA, and FK-binding protein (FKBP), which binds both FK506 and rapamycin. Although the unbound receptors are rotamases (cis-trans peptidyl prolly isomerases) and binding to the respective immunosuppressive ligand inhibits this enzymatic activity, rotamase inhibition alone is not sufficient to explain immunosuppression. There is increasing evidence that the cellular target of both the cyclophilin-CsA and FKBP-FK506 complexes is calcineurin, a Ca++-calmodulin-dependent phosphatase, which is inhibited by these immunophilin-ligand complexes. The inhibition of calcineurin by immunophilin-ligand complexes appears to result in the inhibition of interleukin 2 (IL-2) gene transcription (mediated, in part, by the nuclear factor of activated T cells [NF-AT]) since the concentration of CsA, FK506, or their analogues required to inhibit 50% of NF-AT-mediated IL-2 gene transcription in human T cells is closely correlated with the inhibition of calcineurin in vitro and is increased significantly by either overexpression of calcineurin or a calcineurin catalytic subunit. In contrast to cyclophilin-CsA and FKBP-FK506, the FKBP-rapamycin complex does not interact with calcineurin and does not inhibit IL-2 gene transcription. Rather, this complex appears to interrupt a different T-cell signaling pathway.

Calcineurin, which was first discovered in neural rather than lymphoid tissue, is colocalized with immunophilins in specific brain regions that are involved in the sympathetic control of blood pressure. Therefore, we asked whether inhibition of neuronal calcineurin might play an important physiological role in mediating CsA-induced sympathetic activation and hypertension. We now report that sympathetic activity and arterial pressure in rats are increased not only by CsA...
but also by FK506, which is structurally unrelated to CsA but inhibits the same calcineurin-sensitive T-cell signaling pathway. Studies of neurophysiological signaling pathway and blood sympathetic activation is induced in structure analogues possessing modest each analogue synthesized via supplemental doses of 10 mg/kg). The trachea maintained with a-chloralose (60 mg/kg IV followed by centrifugation and the animal cannulated for infusion of immunophilin ligands, and immunophilin with a 32P-labeled phosphopeptide substrate as previously described.

The rotaform (cis-trans peptidyl prolyl isomerase) activity of the immunophilins was assayed by incubating immunophilin ligands with immunophilin and a test peptide (succinyl-Ala-Leu-Pro-Phe-p-nitroanilide) as previously described.

The inhibition constants, Ki (nanomolar) for the phosphatase activity of calcineurin and Kf for the rotaform activity of immunophilins, were calculated by kinetic analyses as previously described.

**Materials and Methods**

CsA (Sandimmune) was kindly provided by Sandoz Pharmaceuticals Corp, East Hanover, NJ; FK506, FK523 and 15-O-demethyl-FK520, by Fujisawa; and rapamycin, by Wyeth-Ayerst. MeBm2f-CsA and MeAla6-CsA were synthesized as previously described.

**Neurophysiological Studies**

Experiments were performed on female Sprague-Dawley rats weighing 220 to 300 g. Anesthesia was induced with ketamine HCl (80 mg/kg IM) and was maintained with a-chloralose (60 mg/kg IV followed by supplemental doses of 10 mg/kg). The trachea was cannulated, and the animal was artificially ventilated. A femoral vein was cannulated for infusion of immunosuppressive drugs. Arterial pressure was measured via a femoral artery catheter. Renal sympathetic nerve activity was recorded from a branch of the left renal nerve affixed to bipolar platinum electrodes according to the technique of Schad and Seller. Nerve action potentials were detected by a high-impedance probe (model 511, Grass Instruments Co, Quincy, Mass), amplified 20 000-fold (Grass P511 amplifier), filtered (bandwidth, 100 to 2000 Hz), and counted using a window discriminator. Data were recorded continuously on a Gould RS 3600 physiological recorder and stored on FM tape.

**In Vitro Studies**

The phosphatase activity of bovine brain calcineurin was assayed by incubating calcineurin with calmodulin, immunophilin ligands, and immunophilin with a 32P-labeled phosphopeptide substrate as previously described. The rotaform (cis-trans peptidyl prolyl isomerase) activity of the immunophilins was assayed by incubating immunophilin ligands with immunophilin and a test peptide (succinyl-Ala-Leu-Pro-Phe-p-nitroanilide) as previously described.

The inhibition constants, Ki (nanomolar) for the phosphatase activity of calcineurin and Kf for the rotaform activity of immunophilins, were calculated by kinetic analyses as previously described.

**Cellular Studies**

The cellular assay for calcineurin-sensitive signaling inhibition by immunophilin ligands has been described previously. Briefly, a Jurkat T-cell line was stably transfected with a plasmid containing the *Escherichia coli lacZ* gene (which encodes β-galactosidase) attached to the minimal promoter of the IL-2 gene (−72 to +47) linked to a trimer of the NF-AT binding site (−280 to −257 of the IL-2 gene). Cells were activated for 5 hours with 20 ng/mL phorbol 12-myristate 13-acetate and 1.5 µg/mL ionomycin in the presence of immunophilins at various concentrations in a 96-well culture dish (104 cells per well). After activation, cells were harvested by centrifugation at 12 000 rpm in a microcentrifuge and assayed for β-galactosidase activity (used as an index of NF-AT-mediated IL-2 gene transcription). The enzyme assay was as described except for a few modifications. Briefly, 360 µL of the enzyme reaction buffer containing 100 mM Na2HPO4, pH 7.0, 10 mM KCl, 1 mM MgSO4, 0.1% Triton X-100, and 0.5 mM 4-methylumbelliferyl β-D-galactoside was added to the cells and incubated for 1 hour at 25°C. The reaction was stopped by adding 150 µL of stop solution (300 mM glycine and 15 mM EDTA, pH 11.3), and the β-galactosidase activity was determined by the fluorescence measurements (355-nm excitation/460-nm emission). IC50 was calculated as the drug concentration (nanomolar) needed to inhibit 50% of the β-galactosidase activity derived from these activated cells.

**Experimental Protocols**

**Protocol 1: Comparative effects of CsA, FK506, and rapamycin on sympathetic nerve activity and blood pres-**
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FIG 1. Representative experiments from two rats showing multifiber recordings of renal sympathetic nerve activity before and 30 minutes after intravenous infusion of either FK506 (top) or rapamycin (bottom). The frequency and mean amplitude of the bursts of sympathetic activity increased with FK506 but did not increase, and actually decreased, with rapamycin.

Rapamycin

sure. CsA (n=20 rats) was infused intravenously over 20 minutes to a total dose of 5 mg/kg. FK506 (n=22 rats) and rapamycin (n=7 rats) each were infused intravenously over 20 minutes to a total dose of 0.15 mg/kg. These doses of CsA, FK506, and rapamycin have been shown to produce approximately comparable immunosuppression in the rat.38,39 All agents were dissolved in the same vehicle (Cremphor EL, BASF, Aktiengesellschaft, Ludwigshafen, Germany).

Protocol 2: Tachyphylaxis and mutual inhibition in sympathetic nerve responses to CsA and FK506. In 28 additional rats, we tested effects of a first dose of intravenous CsA or FK506 on the subsequent sympathetic responses to a second dose of the same or other drug. The second drug infusion was begun 30 minutes after completion of the first drug infusion. To determine if a single dose of each drug alone would cause a nearly maximal increase in sympathetic activity (which therefore could not possibly be increased further with additional stimulation), we tested the effects of CsA and FK506 on the increase in sympathetic activity evoked by the stimulation of somatic afferents (immersion of the tail in water at 55°C for 60 seconds), a robust nonspecific stimulus to renal sympathetic activity.

Protocol 3: Comparative effects of immunophilin ligands on sympathetic nerve activity and blood pressure with their effects on inhibition of NF-AT-mediated IL-2 gene transcription, phosphatase activity of calcineurin, and rotamase activity of immunophilins. It has been shown that certain modest changes in the structures of

FIG 2. Data from four separate sets of experiments (n=7 rats in each protocol) demonstrating tachyphylaxis (a and b) and mutual inhibition (c and d) in the renal sympathetic nerve responses (expressed in hertz) and blood pressure responses to cyclosporin A (CsA) and FK506.
CsA and FK506 result in marked and specific alterations in the ability of these analogues to inhibit either rotamase and/or calcineurin activity, compared with the parent drugs. The results of these studies with CsA analogues (MeBm\textsubscript{t1}-CsA and MeAla\textsuperscript{8}-CsA) and FK506 analogues (FK523 and 15-O-demethyl-FK520) have been reported recently (Fig 3).\textsuperscript{29} We used these same analogues to examine the strength of correlation between inhibition of the phosphatase activity of calcineurin and activation of sympathetic activity. We recorded renal sympathetic nerve activity and blood pressure during intravenous infusion of the following CsA and FK506 analogues: MeBm\textsubscript{t1}-CsA (n=4 rats) and MeAla\textsuperscript{8}-CsA (n=5 rats) were infused intravenously to a total dose of 5 mg/kg, while FK523 (n=9) and 15-O-demethyl-FK520 (n=6) were infused intravenously to a total dose of 0.15 mg/kg. The neurophysiological studies were performed with the investigators blinded as to the results of the molecular and cellular studies.

Statistical Analysis

Repeated-measures analyses of variance (ANOVA) with Dunnett's post hoc test were used to compare the responses of renal sympathetic nerve activity and blood pressure over time during infusion of drugs. ANOVA with Fisher's protected least significant difference test was used to compare responses between each analogue and its parent immunosuppressive drug. No statistical comparisons were performed between responses to CsA and FK506 because of differences in dosing. In protocol 2, paired t tests were used to compare the differences between preinfusion and postinfusion values of sympathetic nerve activity and blood pressure for the first vs second dose of each drug. Values of \(P<.05\) were considered statistically significant. Data are expressed as mean±SEM.

Results

Sympathetic activity and blood pressure increased markedly with both CsA and FK506 but did not increase and even decreased with rapamycin (Table and Fig 1). Intravenous infusion of vehicle had no effect on either sympathetic nerve activity or blood pressure (Table).

An initial dose of either CsA or FK506 greatly attenuated the increases in sympathetic activity and arterial pressure evoked by a subsequent dose of the same or other drug (Fig 2). An initial dose of either CsA or FK506, however, did not attenuate the increases in sympathetic activity (and arterial pressure) evoked by the stimulation of somatic afferents, a nonspecific test of sympathetic reactivity. Before any drugs were infused, this test caused sympathetic activity to increase from 19±4 to 78±9 Hz (Δ59±5 Hz); after the second drug infusion, it increased sympathetic activity from an already elevated value of 49±10 to 118±13 Hz (Δ69±10 Hz).

Fig 3 illustrates the molecular structures of the different immunophilin ligands.
For analogues of both CsA and FK506, sympathetic activity was strongly associated with inhibition of calcineurin-mediated NF-AT activity, which regulates IL-2 gene transcription (Table and Fig 4). Sympathetic activity and blood pressure increased only with those immunophilin ligands whose immunophilin complexes inhibit the phosphatase activity of calcineurin and calcineurin-mediated inhibition of NF-AT activity. MeBm2t-CsA, whose immunophilin complex is a slightly less potent inhibitor of NF-AT activity than that of CsA, caused a significant increase in sympathetic activity, which tended to be slightly less than that caused by CsA. FK523, whose immunophilin complex causes 60% less inhibition of NF-AT-mediated transcriptional activation than does that of FK506, caused an increase in sympathetic activity that was 60% smaller ($P<.05$) than that produced by the same dose of FK506.

In contrast, sympathetic activity was not increased but rather decreased ($P<.05$) by MeAla$^6$-CsA, 15-O-demethyl-FK520, and rapamycin, three compounds that potently inhibit the rotamase activity of immunophilins but have no effect on either the phosphatase activity of calcineurin or NF-AT-mediated transcriptional activation (Table). Blood pressure was unchanged with MeAla$^6$-CsA and 15-O-demethyl-FK520 but decreased slightly ($P<.05$) with rapamycin. In each experiment in which sympathetic activity decreased, we demonstrated that sympathetic activity could increase normally in response to subsequent challenge with the parent compound, either CsA or FK506 (data not shown).

**Discussion**

In T lymphocytes, calcineurin has been shown to play a pivotal role in mediating CsA- and FK506-induced immunosuppression. The present studies show that the ability of immunosuppressive drugs to cause sympathetic activation and acute hypertension in rats closely parallels their ability to inhibit a specific T-cell signal transduction pathway, a calcineurin-dependent process that is sensitive to CsA and FK506 but not rapamycin. These results implicate an important role for extralymphoid (ie, neuronal) calcineurin in mediating immunosuppressive drug toxicity.

CsA previously has been shown to increase sympathetic vasoconstrictor activity and thereby cause acute hypertension in rats, but the effects of FK506 and rapamycin on sympathetic activity previously have not been described. The finding that sympathetic activity and blood pressure also are increased with FK506, which is structurally unrelated to CsA, demonstrates that sympathetic activation is not a singular side effect of the CsA molecule. The additional finding that sympathetic activity and blood pressure are not increased by rapamycin, an immunophilin ligand that is structur-
ally related to FK506 but does not bind calcineurin, strongly suggests that sympathetic activation is a specific property of only those immunosuppressive drugs whose immunophilin complexes inhibit calcineurin. Cyclophilin-CsA and FKBP-FK506 complexes have been shown to bind competitively to calcineurin, indicating that their binding sites are similar or overlapping. Thus, our demonstration of tachyphylaxis and mutual inhibition in the sympathetic nerve and blood pressure responses to CsA and FK506 strongly suggests that increases in sympathetic activity produced by both drugs are caused by a similar action of these two different immunophilin complexes on a common saturable neuronal target such as calcineurin.

The strongest evidence in support of this hypothesis is that the CsA- and FK506-induced sympathetic activation is attenuated by the same specific structural alterations in these molecules that attenuate their ability to inhibit the phosphatase activity of brain calcineurin and calcineurin-mediated T-cell signaling. Although these data do not prove a causal role for calcineurin in mediating sympathetic overactivity, the strength of the observed correlations is remarkable, since sympathetic activity was measured in intact rats, whereas calcineurin activity was assayed either in vitro or as a function of NF-AT inhibition in T cells. The parallelism between the effects of immunophilin ligands on the immune and sympathetic systems is further suggested by the additional finding that sympathetic activation, like immune suppression, is readily dissociated from the immunophilin-ligand inhibition of rotamase.

An unexpected finding was that sympathetic activity decreased with MeAla-CSA, 15-O-demethyl-FK520, and rapamycin, three immunophilin ligands that have no effect on calcineurin. Although the underlying mechanism is unknown, a possible explanation might be the presence of endogenous immunophilin ligands that, when complexed with immunophiIins, inhibit calcineurin activity and thereby tonically stimulate sympathetic activity. Although these three exogenous ligands do not affect calcineurin directly, they could compete with hypothetical endogenous ligands for immunophilins and thereby reduce the tonic inhibition of calcineurin and the tonic stimulation of sympathetic activity.

The hypothesis that calcineurin is a common cellular target mediating the excitatory actions of both CsA and FK506 on the mammalian sympathetic nervous system is consistent with experiments suggesting that calcineurin dephosphorylates and thereby inactivates voltage-dependent Ca²⁺ channels in molluscan neurons. Because openings of inward Ca²⁺ channels trigger bursts of action potentials in vertebrate ganglion cells, binding of immunophilin-drug complexes to calcineurin might possibly reverse the inhibition of channel activity. Further experiments are needed to test this possibility.

The present findings in an acute experimental animal preparation cannot be extrapolated to make general statements regarding the etiology of chronic hypertension in patients. Clinical studies indicate that hypertension is an important side effect of FK506, as well as CsA, but the clinical toxicity of rapamycin has not yet been examined. On the basis of our experimental findings, we speculate that rapamycin, unlike CsA and FK506, may not cause hypertension in patients.

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Calcineurin-mediated inhibition of T-cell signaling.

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