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Cyclosporin-mediated inhibition of bovine calcineurin by cyclophilins A and B

(calmodulin/immunosuppression)

SELENE K.-H. SWANSON*, TIMOTHY BORN*, LYNNE D. ZYDOWSKY†, HYEONGJIN CHO†, HOWARD Y. CHANG†, CHRISTOPHER T. WALSH†, AND FRANK RUSNAK*‡

*Section of Hematology Research, Mayo Clinic and Foundation, Rochester, MN 55905; and †Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

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ABSTRACT **The Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin is inhibited by the immunosuppressant drug cyclosporin A in the presence of cyclophilin A or B. Of the two isoforms, cyclophilin B is more potent by a factor of 2–5 when either the phosphoprotein [^{32}P]casein or the [^{32}P]phosphoserine [Ser(^{32}P)] form of the 19-residue bovine cardiac cAMP-dependent protein kinase regulatory subunit peptide R_{II}, [Ser(^{32}P)^{15}]R_{II}, is used as substrate. With [Ser($^{32}P^{15}$]R_{II} as substrate, the concentrations of the cyclosporin A·cyclophilin A and cyclosporin A·cyclophilin B complexes, which cause 50% inhibition of calcineurin activity, are 120 and 50 nM, respectively. Lowering the concentration of calcineurin 80% with [^{32}P]casein as substrate lowered the apparent inhibition constant for each complex even further; 50% inhibition of calcineurin was observed at 40 nM for cyclosporin A·cyclophilin A, whereas it was <10 nM for cyclosporin A·cyclophilin B. In all inhibition assays with [^{32}P]casein or [Ser(^{32}P)^{15}]R_{II}, the concentration of calcineurin required for measurable phosphatase activity is such that these complexes behave as tight-binding inhibitors of calcineurin, and steady-state kinetics cannot be used to assess inhibition patterns or K_i values. Limited trypsinization of calcineurin produces a fragment that is still inhibited, indicating that the interaction of cyclosporin·cyclophilin with calcineurin does not require either calmodulin or Ca^{2+}.**

The immunosuppressant cyclosporin A (CsA) blocks T-cell activation by preventing transcription of cytokine genes (1, 2). Although early studies implied that the major transplant drug CsA prevented T-cell activation by binding to calmodulin and inhibiting Ca^{2+}/calmodulin-mediated pathways (3), that proposed interaction is nonspecific (4). The cyclophilins (CyPs), conserved and abundant proteins that possess peptidylproline cis-trans isomerase activity (5, 6), have since been shown to be the site of CsA binding (7, 8). Two distinct classes of CyPs have been identified: the CyPA family, which is distributed throughout the cytoplasm and nucleus, and the CyPB family, which possesses a signal sequence (9) directing it into the endoplasmic reticulum and secretory pathways (F. D. McKeon, personal communication). CsA is a potent inhibitor of the peptidylproline cis-trans isomerase activity of both CyPs, and this has led to the proposal that inhibition of this activity may be important in blocking the immune response (5, 6). However, the ubiquitous nature of the CyPs, the fact that their intracellular concentrations (≥10 μM) (7) exceed the concentrations of CsA (≤1 μM) (2) required for immunosuppression, and results indicating that sensitivity to CsA is mediated by CyP in yeast (10) imply instead that the drug–immunophilin complex interacts with another cellular component. Parallel studies on FK 506, a more potent and structurally distinct immunosuppressant that appears to act through the same pathway, and on rapamycin, which inhibits T-cell activation through a different mechanism, also point to drug–immunophilin complexes as the inhibitory species (11–13).

Evidence that both CsA and FK 506 inhibition affect the same Ca^{2+}-dependent signal transduction pathways showed that both of these immunosuppressants inhibited activation pathways associated with an increase in intracellular Ca^{2+} (14). The recent discovery that calmodulin and the protein phosphatase calcineurin (protein phosphatase 2B or PP2B) are specifically adsorbed on fusion proteins of glutathionyl-*S*-transferase (GST)–CyP and GST–FK 506 binding protein (FKBP) in the presence of CsA or FK 506, respectively, implicates calcineurin as the common target for both CsA and FK 506 inhibition (15, 16).

In the present study, we provide a quantitative assessment of the inhibition of calcineurin by CsA in the presence of either recombinant human CyPA (hCyPA) or CyPB (hCyPB) and demonstrate that the CsA·hCyPB complex is a more potent inhibitor than CsA·hCyPA. For each complex, comparable levels of inhibition are observed when using either the phosphoprotein substrate [^{32}P]casein or the [^{32}P]phosphoserine [Ser(^{32}P)] form of 19-residue bovine cardiac cAMP-dependent protein kinase regulatory subunit peptide II (R_{II}); however, in these studies the concentration of calcineurin required in the inhibition assays is such that CsA·CyP is a tight-binding inhibitor, and the inhibition constants measured represent upper limits of the true K_i values. In experiments aimed at identifying the calcineurin domain(s) necessary for interacting with the CsA·CyP complex, we have determined that a partially trypsinized fragment of calcineurin lacking the autoinhibitory and calmodulin binding domains is still inhibited by the CsA·CyP complexes.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (triethylammonium salt; 3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. ATP (disodium salt), cAMP-dependent protein kinase (catalytic subunit), trypsin, trypsin inhibitor, EGTA, and dephosphorylated α-casein were from Sigma. Bovine calmodulin was purified from bovine brain (17) or purchased from Sigma. CsA was a gift of Sandoz Pharmaceutical, microcystin was from Calbi-

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Abbreviations: CyP, cyclophilin; hCyPA, recombinant human cyclophilin A; hCyPB, recombinant human cyclophilin B; CsA, cyclosporin A; PP1, PP2A, and PP2B, protein phosphatases 1, 2A, and 2B; GST, glutathione *S*-transferase; R_{II}, bovine cardiac cAMP-dependent protein kinase regulatory subunit peptide II; Ser(^{32}P), [^{32}P]phosphoserine; [Ser(^{32}P)^{15}]R_{II}, R_{II} with Ser(^{32}P)-15 in place of Ser-15; FKBP, FK 506 binding protein.

‡To whom reprint requests should be addressed.

ochem, and okadaic acid was from Kamiya Biomedical (Thousand Oaks, CA).

[^{32}P]Casein was prepared (18) to a specific activity of 135 Ci/mol, a yield of 14% based on the activity of the starting [^{32}P]ATP and the amount of protein and radioactivity recovered after dialysis. hCyPA (19) and hCyPB (9) were purified as described. Bovine brain calcineurin was purified to homogeneity (20) and had a specific activity toward [^{32}P]casein of 169 nmol [^{32}P]orthophosphate released per min per mg of protein and a K_m of 3.18 μM, which compare favorably with a previous report (21).

R_{II} peptides containing radioactive or nonradioactive Ser(*P*) [Ser(^{32}*P*) or Ser(^{31}*P*)] in place of Ser-15—namely, [Ser(^{32}*P*)^{15}]R_{II} and [Ser(^{31}P)^{15}]R_{II}—were prepared (22) and purified by HPLC (Vydac Pecosphere, 4.6 × 30 mm). [Ser(^{32}*P*)^{15}]R_{II} and [Ser(^{31}*P*)^{15}]R_{II} were mixed to give ≈50,000–200,000 cpm for each assay.

Protein concentrations were determined with the Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

Methods. *Calcineurin assay with [^{32}P]casein.* [^{32}P]Orthophosphate release from [^{32}P]casein was assayed at 30°C in 50 μl of 40 mM Tris chloride, pH 7.5/0.1 M KCl/6 mM $MgCl_2$/0.1 mM $CaCl_2$/0.1 mg of bovine serum albumin per ml/0.05 mM dithiothreitol/100 nM calmodulin/10 nM calcineurin. All components except substrate were incubated at 30°C for 10 min, and the reaction was started by adding [^{32}P]casein. The reaction was allowed to proceed for 3–6 min (the reaction was linear throughout at all substrate concentrations) and was quenched by the addition of 100 μl of 25% (wt/vol) trichloroacetic acid and 100 μl of bovine serum albumin at 10 mg/ml. After incubation for 5 min on ice, the samples were centrifuged in an Eppendorf microcentrifuge, and a 200-μl aliquot was added to 5 ml of scintillation cocktail and assayed for radioactivity. Rates of [^{32}P]phosphate released were corrected for nonenzymatic hydrolysis by subtracting the amount of ^{32}P released in parallel assays without calcineurin.

Calcineurin assay with ^{32}P-R_{II}. The assay with [Ser(^{32}*P*)^{15}]R_{II} [Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser(^{32}P)-Val-Ala-Ala-Glu] as substrate was identical to that described with [^{32}P]casein except that 50 nM calcineurin was used. The reaction was started with substrate and terminated by the addition of 0.50 ml of 5% trichloroacetic acid containing 0.1 M KH_2PO_4 and water (350 μl), and a slurry of cation-exchange resin (100 μl; Bio-Rad AG50W-X4 H^+ form, 400 mesh, washed successively before use with water, 1.0 M NaOH, 1.0 M HCl, and water) was added to adsorb unreacted [Ser(^{32}*P*)^{15}]R_{II}. The resulting mixture was mixed on a rotator for 10–20 min and centrifuged, and 0.50 ml of supernatant containing free [^{32}P]-orthophosphate was removed and added to 5.0 ml of scintillation cocktail for assay of radioactivity. The V_{max} and K_m values measured in these kinetic assays were 800 nmol·min^{-1}·mg^{-1} and 37 μM, respectively, comparable to previous determinations (22).

Limited proteolysis of calcineurin with trypsin. Trypsinized calcineurin was prepared by incubating calcineurin and trypsin in a final volume of 25 μl in 50 mM Tris chloride, pH 7.0/1–2 μM calcineurin/100 μM $CaCl_2$/0.1% 2-mercaptoethanol/35 nM trypsin at 30°C. After 2 min, 1 μl of trypsin inhibitor (4 μM) was added, and 4-μl aliquots were removed to separate tubes containing inhibitors to assay for phosphate release from [^{32}P]casein as described (18). For the 0 time point of Fig. 3, calcineurin was added to the above proteolysis buffer already containing trypsin inhibitor; for assays in the presence of EGTA, $CaCl_2$ was omitted in the proteolysis and assay buffers.

RESULTS

Inhibition of Calcineurin by CsA with CyPA or CyPB [CsA/CyPA(B)]. Neither hCyPA or hCyPB nor CsA alone inhibited bovine brain calcineurin-catalyzed dephosphorylation of either [^{32}P]casein or 19-residue [Ser(^{32}*P*)^{15}]R_{II} (Table 1, Fig. 1) (22). However, calcineurin was completely inhibited by the combination of either hCyPA or hCyPB with CsA.

Kinetic analyses of calcineurin inhibition by CsA or CyP or a mixture of both were carried out to determine the type and magnitude of inhibition. The data in Table 1 indicate that the complex between CsA and CyP is the inhibitory species, represented by the equilibrium in Eq. **1**:

$$\text{CsA·CyP} + \text{calcineurin} \rightleftharpoons \text{CsA·CyP·calcineurin}. \quad [1]$$

CsA inhibition of the peptidylproline cis-trans isomerase activity of hCyPA ($IC_{50} \leq 10$ nM; ref. 19) and hCyPB (IC_{50} = 84 nM; ref. 9) provides an estimate for the equilibrium in Eq. **2** which must also be considered in the kinetic analysis.

$$\text{CsA} + \text{CyP} \rightleftharpoons \text{CsA·CyP}. \quad [2]$$

At high concentrations of CsA (i.e., 1 μM as in the following assays), the concentration of CsA·CyP approximates that of CyP, and the values for the inhibition of calcineurin obtained below largely reflect the dissociation constant of the drug·immunophilin·calcineurin complex (Eq. **1**).

With [Ser(^{32}*P*)^{15}]R_{II}, the concentrations of hCyPA and hCyPB that caused 50% inhibition of calcineurin were 120 nM and 50 nM, respectively (Fig. 1*A*). Interestingly, these apparent K_i (K_{iapp}) values did not vary substantially as the concentration of [Ser(^{32}*P*)^{15}]R_{II} was varied from 0.5 K_m to 3.0 K_m, implying noncompetitive inhibition by CsA/hCyPA(B) (data not shown). However, since the concentration of calcineurin in these assays (50 nM) was comparable to the concentrations of hCyPA and hCyPB required to cause inhibition, the complex CsA·hCyPA(B) is considered to be a tight-binding inhibitor (23), and a steady-state kinetic analysis cannot be used to determine either the true K_i or whether the inhibition was indeed noncompetitive.

With [^{32}P]casein, the concentration of calcineurin was lowered to 10 nM, and a steady-state analysis was attempted.

Table 1. Calcineurin inhibition by CsA in the presence of either hCyA or hCyB, with [^{32}P]casein, [Ser(^{32}*P*)^{15}]R_{II} peptide, or *p*-nitrophenyl phosphate as substrate

Substrate	Inhibitor	Relative activity, %
[^{32}P]Casein	None	100
	1.0 μM CsA	99
	0.5 μM hCyA	87
	0.4 μM hCyB	74
	1.0 μM CsA + 0.5 μM hCyA	3
	1.0 μM CsA + 0.4 μM hCyB	0
[Ser(^{32}*P*)^{15}]R_{II}	None	100
	10 μM CsA	100
	0.5 μM hCyA	85
	0.4 μM hCyB	83
	10 μM CsA + 0.5 μM hCyA	3
	10 μM CsA + 0.4 μM hCyB	1
p-Nitrophenyl phosphate	None	100
	10 μM CsA	95
	10 μM hCyA	89
	10 μM hCyB	92
	10 μM CsA + 10 μM hCyA	420
	10 μM CsA + 10 μM hCyB	380

Calcineurin was assayed at 30°C by using either [^{32}P]casein or [Ser(^{32}*P*)^{15}]R_{II} peptide as described in text. Phosphate release from *p*-nitrophenylphosphate was followed spectrophotometrically at 405 nm in 1 mM $MnCl_2$/25 mM Mops, pH 7.0 at 23°C. Percent activity is compared in the absence of inhibitors and represents an average of two determinations with [^{32}P]casein and *p*-nitrophenylphosphate and three determinations with [Ser(^{32}*P*)^{15}]R_{II}.

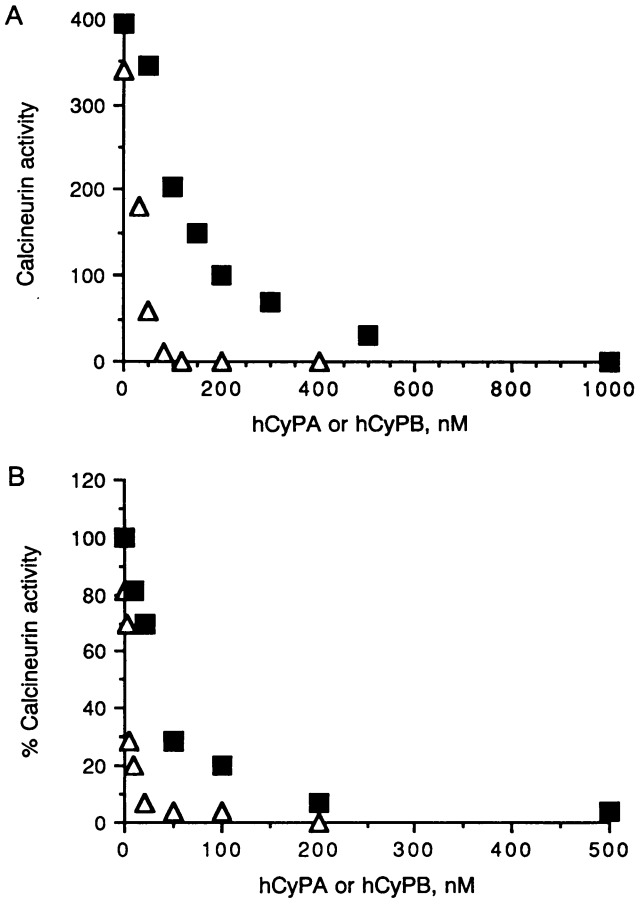

FIG. 1. Inhibition of bovine calcineurin by hCyPA and hCyPB in the presence of CsA. (*A*) Calcineurin activity versus hCyPA (■) or hCyPB (△) with [Ser(^{32}P)^{15}]R_{II} as substrate. Calcineurin activities are expressed as nmol of [^{32}P]orthophosphate released per min per mg of protein. Concentrations of CsA were 10 μM with hCyPA and 1 μM with hCyPB, the R_{II} peptide concentration was 75 μM ($2K_m$), and the calcineurin concentration was 50 nM. (*B*) Calcineurin activity versus hCyPA (■) or hCyPB (△) with [^{32}P]casein as substrate. Calcineurin activities are expressed as percentage of the activity in the absence of CyP and CsA. CsA concentration was 1 μM, casein concentrations were 0.32 μM with hCyPA and 0.83 μM with hCyPB, and calcineurin concentration was 10 nM.

With the concentration of CsA fixed at 1 μM, the complex CsA·hCyPA inhibited calcineurin with a K_{iapp} of 40 nM (Fig. 1*B*). A measurement of the corresponding inhibition of calcineurin by CsA·hCyPB indicated that inhibition occurred at a concentration of hCyPB 80% lower than that observed for CsA·hCyPA; from Fig. 1*B*, the concentration of hCyPB required to inhibit 50% of the activity of calcineurin was found to be 8 nM. Thus, even though the calcineurin concentration was lowered to 10 nM, these assays are still in the tight-binding regime, and the values for inhibition still represent upper limits to the true K_i. Interestingly, CsA·CyP inhibition of calcineurin was partially relieved by increasing [^{32}P]casein concentrations (data not shown), suggesting competitive inhibition.

Microcystin LR and okadaic acid are potent inhibitors of the protein phosphatases 1 and 2A (PP1 and PP2A) but not calcineurin (PP2B) (24). In fact, microcystin resembles CsA in that it is an N-methylated cyclic peptide. The fact that CsA requires CyP to inhibit calcineurin suggests that microcystin might also require a carrier protein (e.g., CyP) for proper presentation as an inhibitor to calcineurin. Neither hCyPA nor hCyPB was able to convert either microcystin LR or okadaic acid into inhibitors of calcineurin, nor did they affect the inhibition of calcineurin by CsA/CyPA(B) (data not shown).

When orthophosphate release from the small, nonphysiological substrate *p*-nitrophenyl phosphate was followed in the presence of Mn^{2+} and calmodulin, calcineurin activity was increased (>4-fold) in the presence of CsA with either hCyPA or hCyPB (Table 1), which also was observed by Schreiber and co-workers for hCyPA (16).

Effect of CsA/hCyP on Trypsinized Calcineurin. The 60-kDa A subunit of calcineurin contains four identifiable domains: an ≈300-residue catalytic domain based on homologies with PP1 and PP2A, a calcineurin B subunit binding domain, a calmodulin binding domain, and an autoinhibitory domain at its carboxyl terminus (Fig. 2) (26–29). In addition, certain isoforms [i.e., the β isoform (30)] contain a polyproline domain at the amino terminus. Limited trypsinization of calcineurin in the absence of calmodulin removed the calmodulin binding and autoinhibitory domains and produced a 43-kDa fragment that retains complete activity no longer stimulated by Ca^{2+} or calmodulin (26, 27).

Trypsinized calcineurin (Fig. 3) was still inhibited by CsA·hCyPA and CsA·hCyPB (Table 2), indicating that the binding site for the CsA·hCyP complex is not part of either the autoinhibitory or calmodulin-binding domains. Furthermore, the addition of EGTA (up to 10 mM) to assays with trypsinized calcineurin had only a slight effect on relieving inhibition. Thus, this interaction between CsA/hCyPA(B) and calcineurin is neither calmodulin- nor Ca^{2+}-mediated.

DISCUSSION

Using CyP-GST and FKBP-GST fusion proteins, Liu *et al.* have recently discovered that calcineurin is specifically adsorbed to these fusion proteins only in the presence of the respective immunosuppressants CsA and FK 506 (16),

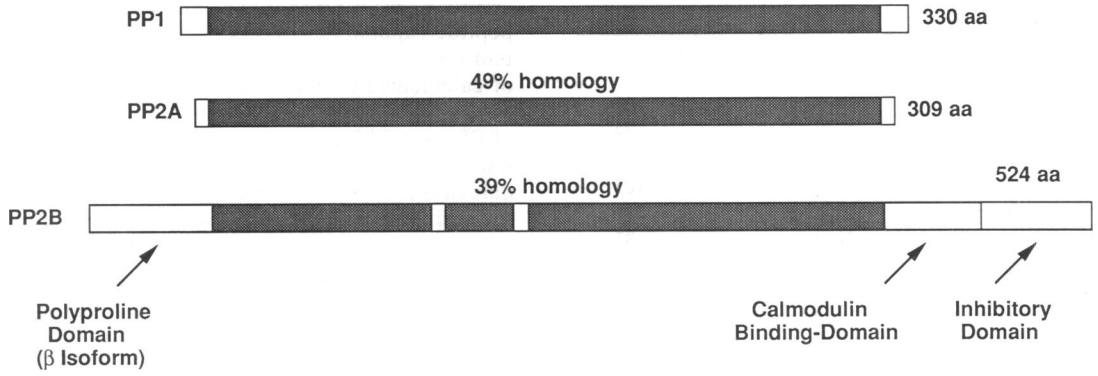

FIG. 2. Schematic representation of the domain structure of PP1, PP2A, and PP2B (from ref. 25). Dark segments represent the conserved catalytic domain. PP2B = calcineurin. aa, Amino acids.

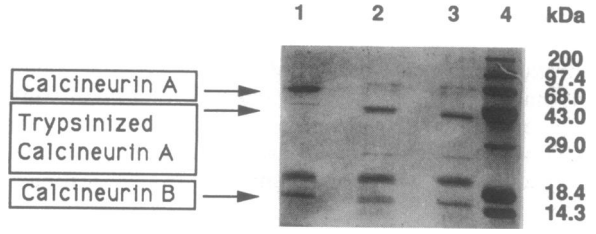

FIG. 3. Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel of trypsinized calcineurin. Lanes: 1, calcineurin 0-min trypsin digest; 2, calcineurin treated with trypsin for 2 min; 3, calcineurin treated with trypsin for 20 min; 4, protein molecular weight standards (masses indicated to the right). Approximately 1.7 μg of calcineurin was loaded in each lane. The arrows indicate the positions of the large (calcineurin A) and small (calcineurin B) subunits of calcineurin. The protein migrating at ≈20 kDa is trypsin inhibitor.

strongly implicating this protein phosphatase as the common *in vivo* target for these immunosuppressant drugs. In this manuscript, we have validated the hypothesis that calcineurin is a target for these drug–immunophilin complexes by assessing the potency of specific isoforms of CyP in the presence of CsA.

With [^{32}P]casein as substrate, the inhibition is potent and appeared competitive with a K_{iapp} of 30–40 nM for hCyPA. With the smaller substrate, [Ser(^{32}P)^{15}]R_{II} peptide, a similar potency is observed, with half-maximal inhibition occurring at ≈120 nM hCyPA, although with this substrate the results suggested a noncompetitive pattern. It can be argued that this substrate-dependent switch from competitive to noncompetitive inhibition reflects the different size, shape, and binding pattern for the large phosphoprotein casein versus the smaller phosphopeptide substrate [Ser(^{32}P)^{15}]R_{II}. The fact that *p*-nitrophenyl phosphate hydrolysis is actually accelerated rather than inhibited (Table 1 and ref. 16) suggests at least some allosteric action by hCyP·CsA complexes that may also extend partially into the phosphatase active site to affect large phosphopeptide and phosphoprotein substrates. However, since the present assay conditions do not represent true steady-state conditions (23), one can debate whether the different inhibition patterns observed for these substrates are artifactual. For example, a switch in inhibition patterns was observed for PP1 with inhibitors 1 and 2 (31). In the tight-binding region, inhibitor 1 acted noncompetitively, while the pattern for inhibitor 2 was that of a mixed-type inhibitor. When the concentration of PP1 was lowered such that [PP1]/[inhibitor] = 0.02 (i.e., steady-state conditions), the inhibition pattern switched to a mixed-type for inhibitor 1 and competitive for inhibitor 2.

Table 2. CsA·CyP inhibition of calcineurin trypsinized in the presence of Ca^{2+} with or without calmodulin

Enzyme	Inhibitor*	Relative activity, %
Native calcineurin (nontrypsinized)		
+Ca^{2+}/+calmodulin	None	100
+Ca^{2+}/−calmodulin	None	27
Trypsinized calcineurin		
+Ca^{2+}/+calmodulin	None	107
+Ca^{2+}/−calmodulin	None	115
+1 mM EGTA	None	83
+Ca^{2+}/+calmodulin	CsA + hCyA	7
+Ca^{2+}/−calmodulin	CsA + hCyA	9
+Ca^{2+}/+calmodulin	CsA + hCyB	2
+Ca^{2+}/−calmodulin	CsA + hCyB	2
+1 mM EGTA	CsA + hCyB	18

Concentrations of CsA and hCyPA were 10 μM and hCyPB was 1.0 μM.

A likely approach, then, to the assays described herein would be to lower calcineurin concentrations further to satisfy the steady-state condition. At present the concentrations of calcineurin in these phosphatase assays are limited to 10–50 nM for each substrate to provide adequate sensitivity for product detection. Subsequent assay development will be required, if possible, to lower calcineurin concentrations 90–98% to assess true K_i values for the CyPA·CsA and hCyPB·CsA complexes.

At the least, the values for K_{iapp} of 40–120 nM for hCyPA and 8–50 nM for hCyPB for the different substrates and assay conditions represent upper limits to the true K_i values. The K_{iapp} value is more likely to decrease further for hCyPB, which inhibits at the same concentration as calcineurin ([I]/[E] ≤ 1), than for hCyPA where the ratio [I]/[E] is larger. With this argument, the vesicular isoform of CyP, hCyPB, seems to be a more effective inhibitor of calcineurin than the cytoplasmic and nuclear variant, hCyPA. Although these data result from the use of a heterogeneous system in which human CyP was inhibitory towards bovine calcineurin, both of these proteins are highly conserved across species (9, 32, 33). Of equal importance in assessing physiological relevance is the fact that isoforms of calcineurin exist (32) and that calcineurin is distributed between both soluble and particulate fractions in both brain (34) and T lymphocytes (35). Given the broad distribution and multiplicity of calcineurin (28–30, 32, 35) and CyPs (9, 19, 33) [as well as FKBPs (36)], it remains to be seen whether inhibition of the protein phosphatase activity of calcineurin isoforms by immunophilin·drug complexes will be of physiological and pharmacological significance in T-cell suppression. The observed variation in CsA·hCyPA versus CsA·hCyPB complexes certainly suggests recognition of both drug and CyP surfaces. The 65% identity of hCyPA and hCyPB coupled with x-ray structure (37, 38) for hCyPA should speed assessment of specificity-conferring residues.

It is noteworthy that the inhibition of calcineurin by CsA·hCyPB occurs at lower concentrations (<10 nM) than are observed for the inhibition of the peptidylproline cis-trans isomerase activity of hCyPB by CsA [≈90 nM (9)]. Since both drug and immunophilin are required for calcineurin inhibition, this observation implies that the association of hCyPB and CsA is tighter in the presence of calcineurin than in its absence. This might explain why certain CsA derivatives such as "1-MeBm$_2$t" (39) are potent immunosuppressants and yet bind poorly to CyP.

The catalytic core of the calcineurin A subunit has high homology to 300-residue stretches of two other protein phosphatases, PP1 and PP2A (Fig. 2) (32, 40). PP1 and PP2A are potently inhibited by the cyclic heptapeptide microcystin LR (IC_{50} < 1 nM), while calcineurin (PP2B) is much less sensitive (24). CsA (41) joins microcystin LR (25) as a cyclic peptide capable of potent (and selective?) inhibition of protein serine/threonine phosphatases. Likewise, the inhibition of calcineurin by the macrocycle FK 506 (in complex with FKBP) may have analogies with the okadaic acid inhibition of PP1 and PP2A. Neither microcystin LR nor okadaic acid inhibit calcineurin in the presence of CyP nor do they have detectable effects on CsA·hCyPA(B) inhibition of calcineurin. This result is intriguing in view of the fact that CsA acts as a chemoprotectant against microcystin LR toxicity *in vivo* (42).

p-Nitrophenyl phosphate hydrolysis requires Mn^{2+} rather than Ca^{2+} for detection (43), which shows that the divalent cation specificity for calcineurin activity is complex. Indeed, when hCyPA·CsA inhibition is measured with Mn^{2+} rather than Ca^{2+}, the inhibition is less potent (data not shown). The finding by Liu *et al.* that the binding of calcineurin to

FKBP-GST (+FK 506) is abolished in the presence of EGTA (16) is contrary to the data presented herein, which shows that EGTA has only a slight effect on relieving calcineurin inhibition by CsA·hCyPA. It is possible that Ca^{2+} (and other divalent metals ions) cause changes that affect both the affinity of calcineurin for CsA·hCyPA and also the specificity and activity towards certain substrates. Calcineurin can also be phosphorylated (44), and not only is its activity highly dependent on the nature of the divalent cation, but also substrate-selective stimulation by certain phospholipids has been reported (45). It is unknown at present whether these influence the inhibition by CsA·CyPA(B). Furthermore, CsA binds, albeit nonspecifically, to calmodulin (4), so inhibition profiles require careful specification of reaction conditions, including the choice of substrate.

These results show that *in vitro*, CsA can exert its effects by inhibiting the dephosphorylation of as-yet-unidentified proteins. Recently, it has been determined that both CsA and FK 506 block the translocation to the nucleus of a cytoplasmic form of the T-cell transcription factor NF-AT (46). Determination of specific cellular phosphoprotein substrates for calcineurin, such as components of transcription factors or anchor proteins (46, 47) or proteins involved in membrane trafficking, may be fruitful for deciphering signal-transduction pathways.

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