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ROLES OF PEPTIDYL–PROLYL *CIS–TRANS* ISOMERASE AND CALCINEURIN IN THE MECHANISMS OF ANTIMALARIAL ACTION OF CYCLOSPORIN A, FK506, AND RAPAMYCIN

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Abstract—The immunosuppressive peptide cyclosporin A inhibits the growth of malaria parasites *in vitro* and *in vivo*, but little is known about its mechanism of antimalarial action. The immunosuppressive action of cyclosporin A is believed to result from binding of the drug to cyclophilins (intracellular peptidyl–prolyl *cis–trans* isomerases), and inhibition of the protein phosphatase calcineurin by the cyclosporin A–cyclophilin complex. Two immunosuppressive macrolides, FK506 and rapamycin, bind to a distinct isomerase, FKBP12, and the FK506–FKBP complex also inhibits calcineurin. Calcineurin itself is apparently involved in signal transduction between the T-cell membrane and nucleus, and its inhibition blocks T-cell activation. Rapamycin inhibits a later step in T-cell proliferation. Peptidyl–prolyl *cis–trans* isomerase activity was detected in extracts of *Plasmodium falciparum*. It was completely inhibited by concentrations of cyclosporin A above 0.1 μM, but not by FK506 or rapamycin, and probably represented one or more cyclophilins. Comparison of the antimalarial and anti-isomerase activities of a series of cyclosporin analogues failed to reveal a correlation between the two properties. Cyclosporin A and its more active 8′-oxymethyl-dihydro-derivative, in combination with the cyclophilin-containing *P. falciparum* extract, inhibited the protein phosphatase activity of bovine calcineurin. Therefore inhibition of a putative *P. falciparum* calcineurin by a complex of CsA and cyclophilin might be responsible for the antimalarial action of the drug. The most active cyclosporin, however, was a 3′-keto-derivative of cyclosporin D (SDZ PSC-833) which inhibited *P. falciparum* growth with a 50% inhibitory concentration (IC_{50}) of 0.032 μM (compared with 0.30 μM for cyclosporin A), but was a poor inhibitor of the parasite isomerase. 3′-Keto-cyclosporin D has negligible immunosuppressive activity, but it strongly inhibits the P-glycoprotein of multi-drug resistant mammalian tumour cells. FK506 and rapamycin were also active antimalarials (IC_{50} of 1.9 and 2.6 μM, respectively) but in the absence of detectable FKBP in *P. falciparum* extracts, their mechanisms of antimalarial action remain unclear.

Key words: cyclophilin; protein folding; protein serine/threonine phosphatase; immunosuppressive drugs; malaria; *Plasmodium falciparum*

CsA† is a fungal metabolite with potent immunosuppressive properties. It has been used extensively to prevent rejection of organ transplants and to control certain autoimmune diseases [1]. It is a hydrophobic, cyclic undecapeptide. Its unexpected antimalarial activity was first reported on blood-stage *Plasmodium berghei* and *P. chabaudi* in mice [2], and was later confirmed with *P. yoelii* [3]. Antimalarial activity *in vitro* was described with the human malaria parasite *P. falciparum* [3]. In the rodent malaria models as well as in *P. falciparum*, CsA apparently acts directly on the parasite. It is most effective administered when parasitemia is already established, and retains its activity in nude Balb/c mice [2]. Parasites isolated from recrudescent infections are frequently more resistant to CsA, and resistant *P. yoelii* have reduced virulence [3] and resistant *P. berghei*, reduced infectivity for their mosquito vectors [4]. Minimum effective doses for rodent malaria are in the range of 3–25 mg/kg/day [2–6]. CsA also reduces the severity of *P. falciparum* malaria in owl monkeys, but the doses used (25–150 mg/kg/day) may have resulted in toxicity [5]. CsA is also effective against a variety of other parasites, most notably *Schistosoma* spp., although in most parasite species suppression of immunopathology contributes to the efficacy of the drug in addition to or to the exclusion of antiparasitic action [7].

Little is known about how CsA inhibits the growth of malaria parasites. In *P. berghei* malaria, the ring and schizont stage parasites of the asexual erythrocytic cycle disappear first in response to CsA treatment, followed by the trophozoites, but the

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† Abbreviations: Cs, cyclosporin; CsA, cyclosporin A; CsD, cyclosporin D ([Val^2]-Cs); CsG, cyclosporin G ([Nva^2]-Cs); CsH, cyclosporin H ([D-$MeVal^{11}$]-Cs); MeBmt, (4*R*)-4[(E)-2-butenyl]-4:*N*-dimethyl-L-threonine; Abu, L-α-aminobutyric acid; Sar, sarcosine; Nva, L-norvaline; PPIase, peptidyl–prolyl *cis–trans* isomerase; MDR, multi-drug resistance; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid; IC_{50}, 50% inhibitory concentration; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; Dap, D-diaminopropyl; Dab, D-diaminobutyryl.

gametocytes are relatively resistant [4]. Maturation of *P. falciparum* schizonts *in vitro* is particularly susceptible [8]. Dansylated CsA is concentrated inside infected erythrocytes, and [^{3}H]photoaffinity-labelled CsA is accumulated in the digestive vacuole and distributed around the cytosol of *P. falciparum* [9]. The digestive vacuole also sustains ultrastructural damage after 6 hr of exposure to 4.2 μM CsA. CsA has been proposed to act as a protease inhibitor [10] or a calmodulin inhibitor [9], but no direct evidence for either hypothesis has been published.

We decided to investigate the mechanism of antimalarial action of CsA in light of the startling advances that have been made recently in elucidating its immunosuppressive mechanism. The first of these was that the major binding proteins in lymphoid cells for CsA (the cyclophilins [11]) and for the macrolide immunosuppressant FK506 [12] (FKBP12), though unrelated in sequence, share PPIase activity [13–16]. PPIase (EC 5.2.1.8) catalyses the *cis–trans* isomerisation of peptidyl–prolyl bonds in peptides and proteins and plays a role in protein folding *in vitro* and probably *in vivo* [17]. Both cyclophilin and FKBP protein families are ubiquitous, and different isoforms of each reside in different subcellular locations. CsA and FK506 do not, however, appear to act directly as a result of inhibition of PPIase; rather, complexes of CsA–cyclophilin and FK506–FKBP12 inhibit the activity of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, *in vitro* [18] and in intact T-lymphocytes [19]. The drugs alone do not inhibit calcineurin. The bulk of evidence already suggested that CsA (and FK506) acts primarily on the Ca^{2+}-dependent signal transduction pathway of T-cell activation that lies between the T-cell membrane, with its receptor and associated signalling components, and the nucleus, where expression of lymphokine genes is inhibited by the drugs [20, 21]. Calcineurin is now believed to be a component of this pathway [22, 23], and its inhibition by the drug–protein complexes may prevent dephosphorylation and transport into the nucleus of transcription factors such as NF-AT that regulate lymphokine gene transcription [24, 25]. Rapamycin is a macrolide immunosuppressant that binds FKBP but acts on a later stage of T-cell proliferation. Whether or not it is complexed with FKBP, rapamycin does not inhibit calcineurin [21].

We now demonstrate PPIase activity that is inhibitable by CsA, but not by FK506 or rapamycin, in extracts of *P. falciparum*. This suggests that there are one or more cyclophilins in the parasite, but no demonstrable FKBPs. Our results with several other cyclosporins showed that there was no correlation between PPIase inhibition and antimalarial activity. CsA and its 8′-*O*-methyl-dihydro-MeBmt[1] derivative were capable, however, of inhibiting bovine calcineurin when combined with the PPIase-containing parasite extract. We also show that the 3′-keto-MeBmt[1] derivative of CsD, a known reverser of MDR in tumor cells, is the most active antimalarial cyclosporin found so far.

MATERIALS AND METHODS

Parasites and extracts. Plasmodium falciparum FCH5.C2, a cloned subline of FCH5/Tanzania [26] was maintained in erythrocyte culture by standard methods [26]. Parasites were freed from mixed-stage cultures of 8–15% parasitemia by treatment with 0.0015% saponin for 20 min at 4°. Washed parasite pellets were suspended in 53 μL of storage solution (50 mM HEPES–Na^+[pH 8.0 at 0°], 100 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 1 μg/mL pepstatin A, 20 μg/mL leupeptin [all from Sigma, Buchs, Switzerland], 10% v/v glycerol) per 10 mL culture (5% haematocrit). The suspensions were divided into small amounts, quick-frozen, and stored at −70°. To prepare *P. falciparum* extract, a portion of parasite suspension was thawed, mixed with Triton X-100 to a concentration of 0.5% v/v, incubated for 30 min on ice, then centrifuged at 15,000 *g* in an Eppendorf centrifuge for 10 min at 4°. The supernatant ("*P. falciparum* extract") was used for PPIase and calcineurin assays as described below.

Compounds. Cyclosporins and rapamycin were a kind gift of Sandoz Pharma AG (Basel, Switzerland) and FK506 of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Cyclosporins were prepared as 10 mg/mL stock solutions in 95% ethanol/5% Tween 40, rapamycin in methanol, and FK506 (5 mg/mL) in dimethylsulphoxide. Dilutions were prepared freshly on the day of the experiment. In all the drug experiments, corresponding amounts of solvent alone were added to the controls.

PPIase assay. Spectrophotometric determination of PPIase activity was done by the method of Kofron *et al.* [27]. Cuvettes contained (unless otherwise stated) 648 μL of 50 mM HEPES–Na^+ (pH 8.0 at 0°), 100 mM NaCl, 7.5 μL of *P. falciparum* extract, and (added just before starting absorbance measurement) 75 μL of 60 mg/mL chymotrypsin (in 1 mM HCl) and 19 μL of 4.0 mM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma: in trifluoroethanol/470 mM LiCl). Succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (Bachem, Bubendorf, Switzerland) was used where indicated at the same concentration. This gave a final concentration of 100 μM peptide substrate, of which 45–80% was in the *cis*-conformation (Fig. 1 and data not shown). The cuvette was incubated for 3 min at 0° (at which temperature the nonenzymic thermal isomerisation is suppressed [27]) while the change in absorbance at 390 nm was monitored. *Trans*-peptide is instantaneously cleaved by chymotrypsin to give the coloured *p*-nitroaniline product. Thus the change in absorbance reflects the rate of *cis–trans* isomerisation by PPIase. In some experiments, appropriate dilutions of drugs or solvents in HEPES/NaCl were pre-incubated for 5 min at 0° with *P. falciparum* extract in the cuvette, before adding chymotrypsin and substrate. In all assays the final volume was 750 μL. First order rate constants for the conversion of *cis*- to *trans*-substrate [13] were calculated by plotting the natural logarithm of the concentration of *cis*-substrate remaining against time using the Cricket-Graph programme on a Macintosh computer.

Growth inhibition assay. Concentrations of drugs required to inhibit parasite growth by 50% after 48 hr (IC_{50}) were determined by the method of [G-^{3}H] hypoxanthine incorporation [28], using cultures

of haematocrit 1.0% and initial parasitemia 0.8%, and the data were analysed graphically.

Protein serine/threonine phosphatase assay. Phosphatase assays were done by the method of Tallant and Cheung [29], using as the substrate casein labelled with [γ-^{32}P]ATP by the recombinant *P. falciparum* calcium-dependent protein kinase.* Labelled casein was separated from unincorporated ATP on columns of Sephadex G50 (Pharmacia, Dübendorf, Switzerland). Phosphatase reactions contained 50 mM Tris–HCl pH 7.0, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, 1 mg/mL bovine serum albumin, 100 nM calmodulin (Sigma), and where appropriate 10 nM bovine brain calcineurin (Sigma) and/or 4.0% *P. falciparum* extract (supplemented with $MgCl_2$ to 2 mM). Reactions lacking extract contained a corresponding amount of storage solution, Triton X-100, and $MgCl_2$. Okadaic acid (Böehringer-Mannheim, Mannheim, Germany) at a concentration of 500 nM (which is sufficient to inhibit protein phosphatases PP1 and PP2A but not calcineurin and certain other phosphatases [30]) and drugs (0.3 μM), solvent, or EGTA (5 mM) were then added where appropriate and the reaction tubes were preincubated for 10 min at 30°. Reactions were started by the addition of 100,000 cpm of [^{32}P]casein. They were stopped with trichloroacetic acid after the times indicated and the phosphate released was determined by liquid scintillation counting of cold trichloroacetic acid-soluble material as previously described [29].

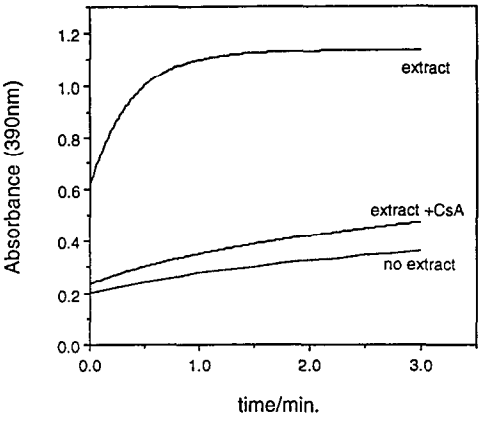

Fig. 1. Representative curves showing PPIase activity of a *P. falciparum* extract and its inhibition by CsA. Release of *p*-nitroaniline from the peptide substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide following *cis*→*trans* conversion of the Ala–Pro bond was monitored spectrophotometrically as described in Materials and Methods. The starting absorbance (typically 0.25–0.7) represented the proportion of substrate molecules already in the *trans*-conformation at the start of the experiment. The gradual increase in absorbance in the absence of extract is due to slow thermal isomerisation. In the presence of extract, the curve plateaus at the maximum absorbance due to exhaustion of the substrate. CsA was added where indicated to a concentration of 0.083 μM.

RESULTS

Demonstration of PPIase activity in P. falciparum

We first sought to explain the potent antimalarial activity of CsA by looking for a *P. falciparum* homologue of the major CsA-binding protein, cyclophilin [11]. Triton X-100 extracts of parasites were therefore tested for the presence of PPIase, the enzymic activity characteristic of cyclophilins and FKBPs, by spectrophotometric assay [27]. *P. falciparum* extracts at 1.0% of the assay volume catalysed the *cis*→*trans* conversion of an Ala–Pro bond in a test substrate with a first order rate constant (k) in the range 0.02–0.06 sec^{-1} (Fig. 1). The undiluted extracts, which contained ~4 mg/mL protein, had thus the catalytic activity of 130–400 nM recombinant human cyclophilin [27, 31]. In the absence of extract, the nonenzymic background rate was approximately $k = 0.002$ sec^{-1}. When the background rate was subtracted, the rate constant was roughly proportional to the amount of extract added (data not shown). Concentrations of CsA in the order of 0.1 μM or more inhibited the PPIase activity completely (Figs. 1 and 2), indicating that the PPIase present was likely to belong to the cyclophilin family. By contrast, neither FK506 nor rapamycin inhibited the *P. falciparum* enzyme at the concentrations tested (Table 1). When the extract was assayed using a peptide with a Leu–Pro instead of an Ala–Pro bond, according to the substrate preference of FKBPs [17], the results were the same

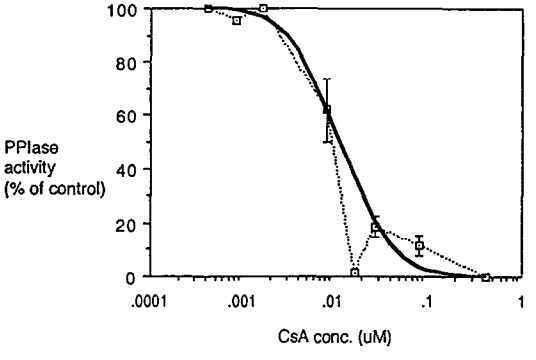

Fig. 2. Effect of CsA concentration on PPIase activity. PPIase in *P. falciparum* extracts was assayed in the presence of various concentrations of CsA, and rate constants calculated, as described in Materials and Methods. Vertical bars for replicate determinations indicate standard errors.

(data not shown). The *P. falciparum* extract appeared, therefore, to contain one or more cyclophilins but no FKBPs, unless the parasite FKBPs were inactivated by the extraction or by the assay conditions used. Extracts of uninfected erythrocytes with a protein concentration of ~1 mg/mL gave a rate constant of ~0.006 when tested at 1.0% concentration in the same assay. This was probably due, at least in part, to the erythrocyte cyclophilin described previously [31a]. The eryth-

* Y. Zhao, R. M. Franklin and B. Kappes, manuscript submitted.

Table 1. Inhibition of *P. falciparum* growth and PPIase activity by CsA, cyclosporin derivatives, FK506 and rapamycin

Compound	*P. falciparum* $IC_{50}/\mu M$¶ growth	PPIase	Immunosuppressive activity‖
[3′-keto-MeBmt[1]]-CsD‡	0.032†††	1†††	*
[8′-OMe-dihydro-MeBmt[1]]-CsA	0.089†	0.006	**
[dihydro-MeBmt[1]]-CsD	0.11	0.02	**
[8′-azido-dihydro-MeBmt[1]]-CsA	0.14	0.01	**
[3′-keto-MeBmt[1]]-CsA	0.23	0.6†††	*
CsD ([Val[2]]-Cs)	0.26	0.02	**
CsA	0.30	0.01	***
[Ile[2]]-Cs	0.31	0.03	*
[MeVal[4]]-CsA	0.33	0.008	*
[dihydro-MeBmt[1]] [SMe-Sar[3]]-CsD	0.33	0.02	***
CsG ([Nva[2]]-Cs)	0.35	0.02	***
[D-MeAla[3]]-CsD	0.46	0.008	***
[OAc-MeBmt[1]]-CsA	0.74†	>4†††	*
[D-MeSer[3]]-CsA	0.97†	0.004	***
CsH ([D-MeVal[11]]-Cs)	1.2††	>4†††	*
[*N*-phenylthiourea-iso-MeBmt[1]]-CsA§	1.2††	0.5†††	*
FK506	1.9††	>2.5†††	****
Rapamycin	2.6†††	>1†††	****

¶ Growth and PPIase activity were assayed at a range of drug concentrations, by [^{3}H]hypoxanthine incorporation and spectrophotometry, respectively, as described in Materials and Methods. 50% inhibitory concentrations (IC_{50}) were determined graphically.

‖ Immunosuppressive activity in mixed lymphocyte reactions [43]: *, little or none; **, less than CsA; ***, equal to CsA; ****, more than CsA [R. Wenger, personal communication].

†, ††, ††† Different from CsA by Student's *t*-test at: †$P < 0.1$, ††$P < 0.01$, †††$P < 0.001$. All others $P > 0.1$.

‡ The side-chain carbon atoms of MeBmt[1] are numbered 3′ to 8′ [44].

§ Iso-CsA has an ester bond between the 3′-OH of MeBmt[1] and the carbonyl of MeVal[11] instead of the usual peptide bond [44].

rocytic PPIase activity was not high enough, however, to have contributed significantly as a contaminant to the *P. falciparum* PPIase activity that was measured.

Antimalarial activity and PPIase inhibition by CsA derivatives, FK506 and rapamycin

To determine whether CsA might inhibit *P. falciparum* growth directly as a result of inhibition of PPIase, i.e. by blocking essential protein folding reactions, IC_{50} for (i) parasite growth and (ii) PPIase were compared for CsA and a variety of other naturally-occurring, synthetic, or semi-synthetic cyclosporins (Table 1). CsA had an IC_{50} of 0.3 μM for *P. falciparum* growth and 0.01 μM for PPIase, indicating that the enzyme would be fully inhibited by concentrations below those required for antimalarial activity (Fig. 2 and Table 1). Seventeen other cyclosporins were also tested (Table 1 and data not shown). All had significant antimalarial activity, and several were more active than CsA.

Some of the cyclosporins different from CsA in the 1-position had antimalarial activity superior to CsA. Although both [3′-keto-MeBmt[1]]-CsA and CsD ([Val[2]]-Cs) were no more active than CsA, [3′-keto-MeBmt[1]]-CsD (SDZ PSC-833) was about ten-fold more active, and is the most active antimalarial cyclosporin so far described. Its IC_{50} of 0.032 μM compared favourably with those of two established antimalarial drugs, chloroquine ($IC_{50} = 0.018\ \mu M$) and arteether ($IC_{50} = 0.034\ \mu M$), tested against the same strain. [3′-keto-MeBmt[1]]-CsD is also the most active cyclosporin so far described in reversal of multi-drug resistance (MDR) in tumour cells [32, 33]. Among the changes that reduced the antimalarial activity of CsA were epimerisation of residue 11 (CsH) and acetylation of the hydroxyl group in the side chain of MeBmt in position 1. The anti-PPIase activities of the cyclosporins did not correlate well with their antimalarial activities (Table 1). The most active antimalarials, however, all inhibited PPIase with IC_{50}s less than 0.04 μM, except the 3′-keto-derivatives of CsA and CsD.

The macrolide immunosuppressants FK506 and rapamycin also had significant antimalarial activity, though inferior to that of CsA (Table 1).

Effect of cyclosporins in combination with P. falciparum *extract on bovine calcineurin*

Although protein phosphatase activity was easily demonstrated in *P. falciparum* extracts, it was not significantly inhibited by adding EGTA to or by omitting calmodulin from the reactions, and was ~80% inhibited by 500 nM okadaic acid. This indicated that most of the parasite phosphatase activity came from enzymes other than calcineurin, while not ruling out the presence of calcineurin in the parasite. To determine whether a complex of CsA and cyclophilin might be the toxic agent in *P. falciparum*, as in T-cells, we tested the ability of CsA and *P. falciparum* extract alone and in

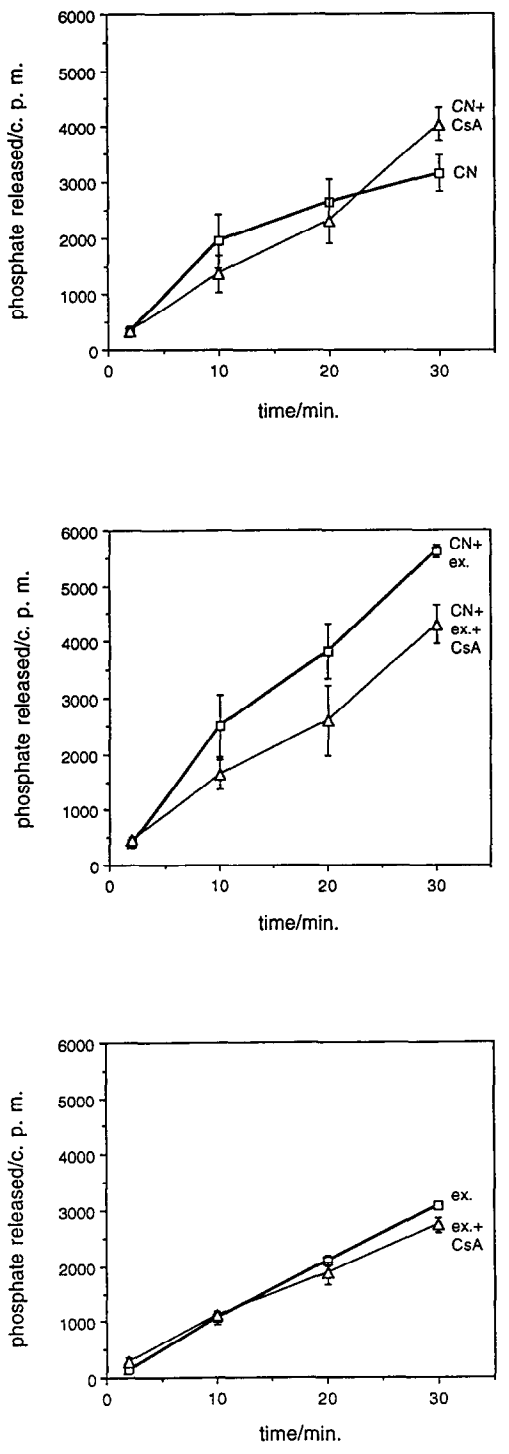

Fig. 3. Effect of CsA, alone and in combination with *P. falciparum* extract, on phosphatase activity of bovine calcineurin. $^{32}P_i$ released from labeled casein was assayed in the presence of 500 nM okadaic acid as described in Materials and Methods. Points are means of three experiments using the same batch of [^{32}P]casein, and vertical lines represent standard errors. Panel A, 10 nM calcineurin alone; panel B, calcineurin plus 4.0% *P. falciparum* extract; panel C, extract alone. Assays were done in parallel in the presence (triangles) or absence (squares) of 0.3 μM CsA.

combination to inhibit the phosphatase activity of bovine brain calcineurin. The experiments were carried out in the presence of 500 nM okadaic acid to reduce the background phosphatase activity of the extract, while having little effect on the calcineurin. While CsA did not significantly reduce the phosphatase activity of either calcineurin alone (Fig. 3A) or *P. falciparum* extract (Fig. 3C), the combination of calcineurin and extract had lower activity in the presence of CsA (Fig. 3B). Assuming that CsA in combination with some component of the extract (e.g. cyclophilin) was reducing the activity of calcineurin rather than *vice versa*, subtracting the values in Fig. 3C from those in Fig. 3B indicates a 49–52% inhibition of calcineurin by 0.3 μM CsA (which was the IC_{50} of the drug for *P. falciparum* growth) plus extract. The activity of calcineurin was not reduced by CsA in combination with boiled extract (data not shown).

The effects of several cyclosporins of differing properties (Table 1) and of FK506 were studied in the same way. In the absence of drug, the phosphatase activity of calcineurin-plus-extract was equal to or slightly lower than the sum of the activities of calcineurin alone and extract alone (Fig. 4). The same was seen when [3′-keto-MeBmt[1]]-CsD, [3′-keto-MeBmt[1]]-CsA, [OAc-MeBmt[1]]-CsA, [D-MeSer[3]]-CsA, or FK506 were added. CsA and [8′-OMe-dihydro-MeBmt[1]]-CsA (B-5-49 [7]), by contrast, gave phosphatase activities in the calcineurin-plus-extract assays that were well below the sums of the calcineurin alone and extract alone assays. Using the assumption mentioned above and subtracting the "extract alone" values, the data indicated 67% and 61% inhibition of calcineurin activity by CsA and its OMe-dihydro-derivative (plus extract), respectively. In summary, the compounds that inhibited PPIase by less than 50% at 0.3 μM had no effect (in combination with *P. falciparum* extract) on bovine calcineurin, possibly because their weaker cyclophilin-binding failed to generate a toxic level of drug–cyclophilin complex. Two of the three potent PPIase inhibitors did (in combination with extract) inhibit calcineurin and a third ([D-MeSer[3]]-CsA) did not. The lower antimalarial activity of the [D-MeSer[3]]-CsA might therefore be explained by the low affinity of the drug–cyclophilin complex for the *P. falciparum* calcineurin, if the latter exists.

DISCUSSION

Scheibel and co-workers [10], using CsA and six of its variants whose structures were not divulged, first showed that the order of antimalarial efficacy of cyclosporins did not match their order of immunosuppressive efficacy. This suggested that cyclosporin derivatives could be found with antimalarial activity equal to or greater than that of CsA, but without immunosuppressive activity. We have confirmed and extended this finding with a series of cyclosporin variants, mainly at the 1-, 2- and 3-positions. The following observations suggested that antimalarial and immunosuppressive efficacy did not match: (i) all of the cyclosporins tested had significant antimalarial activity, whereas some (e.g. [Ile[2]]-Cs, [OAc-MeBmt[1]]-CsA, [MeVal[4]]-

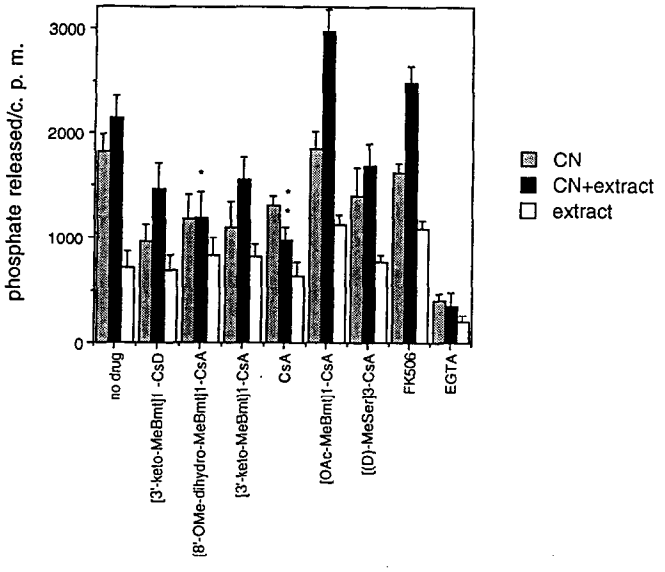

Fig. 4. Effects of cyclosporins, FK506, and EGTA, alone and in combination with *P. falciparum* extract, on phosphatase activity of bovine calcineurin. ^{32}P released from labeled casein was assayed after 20 min incubation. Bars show the means of three or more duplicate experiments each with the same batch of [^{32}P]casein, and vertical lines represent standard errors. Cyclosporins and FK506 were present at 0.3 μM and EGTA at 5 mM where indicated. CN, calcineurin. *, "CN-plus-extract" significantly less than the sum of "CN" and "extract" at $P = 0.1$; ** at $P < 0.05$; all others $P > 0.1$. Experiments with different drugs/solvents were done separately, in contrast to the experiments presented in Fig. 3.

CsA) have essentially no immunosuppressive activity (Table 1); and (ii) certain structural alterations that render the CsA molecule less immunosuppressive (e.g. oxidation of the 3'-OH of MeBmt[1] to a keto group, substitution of Val[2] for Abu[2] [11]) did not reduce antimalarial activity (Table 1). The most active antimalarial compound, [3'-keto-MeBmt[1]]-CsD (SDZ PSC-833, [32]) had an IC_{50} that was ten-fold lower than CsA, and comparable to established antimalarial agents. This compound has already been tested successfully *in vivo* as a reverser of MDR in cancer [34]. It appears that this or a closely-related compound would be a promising antimalarial candidate. We have not attempted comprehensive structure–activity analysis here, but the testing of a wider series of MeBmt[1] derivatives of CsA, CsD and CsG, in addition to exploration of changes in other residues (especially 6 and 8: see below) might reveal compounds even more active against *P. falciparum* than [3'-keto-MeBmt[1]]-CsD.

Given our observation that *P. falciparum* contains CsA-inhibitable PPIase activity, there are two obvious hypotheses for the mechanism of antimalarial action of CsA. One is that inhibition of parasite growth is a direct result of inhibition of PPIase, i.e. essential protein folding reactions are inhibited. Another, by analogy with the current model of immunosuppressive action of CsA, is that CsA combines with parasite PPIase (cyclophilin) to inhibit an essential parasite target, such as a calcineurin homologue. Before the discovery of the PPIase activity of cyclophilin, Quesniaux *et al.* compared the immunosuppressive properties of some 50 cyclosporins with their cyclophilin binding [35]. The correlation was good, with a few exceptions that were ascribed to differences in uptake rates or metabolic transformation. When it later became clear that cyclophilin-binding/PPIase inhibition did not directly cause immunosuppression by CsA [20], the anomalous compounds were proposed (in their complexes with cyclophilin) to have widely different affinities for the true target, which is now believed to be calcineurin. This was confirmed for [$MeBm_2t^1$]-CsA, which has weaker binding to cyclophilin (K_i = 500 nM) than does CsA (K_i = 5–6 nM), but similar immunosuppressive potency: the [$MeBm_2t^1$]-CsA–cyclophilin complex had 2–3-fold higher affinity for purified calcineurin than its CsA–cyclophilin counterpart [36, 37]. Conversely, the drug–protein complexes of the strongly cyclophilin-binding but weakly immunosuppressive [MeAla[6]]-, [Dap[8]]- and [Dab[8]]-CsA derivatives had much lower affinity for calcineurin [36, 37]. Our comparison of the IC_{50}s of cyclosporins for (i) growth and (ii) PPIase of *P. falciparum* shows little or no correlation between the two (Table 1). The 3'-keto-MeBmt[1] derivatives of CsA and CsD, which were among the strongest antimalarials, were weaker PPIase inhibitors than CsA. Conversely, [D-MeSer[3]]-CsA (Table 1), and a 3- and 8-position derivative with IC_{50}s of 0.89 μM for growth and 0.0009 μM for PPIase (not shown in Table 1), were weaker antimalarials but were among the strongest PPIase inhibitors. These findings conflict with the idea that the primary mechanism of antimalarial action of cyclosporins is on protein folding. This idea cannot be ruled out, however, because we know nothing of the rates of uptake of the different cyclosporins into *P. falciparum* cells.

Turning to the second hypothesis, we have tested the ability of selected cyclosporins, in combination with cyclophilin-containing parasite extract, to inhibit bovine calcineurin. Three of the compounds tested were strong inhibitors of PPIase and might be expected to form significant concentrations of cyclosporin–cyclophilin complex: CsA, [8′-OMe-dihydro MeBmt[1]]-CsA (which had stronger antimalarial activity than CsA), and [D-MeSer[3]]-CsA (which was a weaker antimalarial than CsA). The first two apparently inhibited bovine calcineurin, but the third did not (Fig. 4). We might explain the weaker antimalarial activity of [D-MeSer[3]]-CsA by analogy with the [MeAla[6]]-, [Dap[8]]- and [Dab[8]]-derivatives mentioned above, i.e. by lower affinity of the [D-MeSer[3]]-CsA–cyclophilin complex for a putative parasite calcineurin. We also tested in the calcineurin assay three cyclosporins that were poor inhibitors of PPIase. These were [3′-keto-MeBmt[1]]-CsD (antimalarial activity superior to CsA), [3′-keto-MeBmt[1]]-CsA (about equal to CsA) and [OAc-MeBmt[1]]-CsA (inferior to CsA). As expected, none had any effect on calcineurin. Therefore, if we set aside for the moment the 3′-keto-derivatives, we might explain the mechanism of antimalarial action of cyclosporins by formation of a toxic cyclosporin–cyclophilin complex that inhibits a parasite homologue of calcineurin. The unusually high susceptibility of *P. falciparum* cells to CsA compared with mammalian cells might be the result of (i) higher binding affinities of the parasite cyclophilin for CsA and/or the parasite calcineurin for the CsA–cyclophilin complex (due to differences in amino acid sequence or intracellular environment), (ii) higher cyclophilin : calcineurin ratios in parasite cells, (iii) more rapid uptake of CsA into parasite cells, or (iv) an essential rather than facultative role of calcineurin in parasite growth and survival. Several further experiments will be needed, however, to show whether this cyclophilin–calcineurin mechanism is significant in *P. falciparum*: firstly, the purification of cyclophilin from parasite extract and demonstration that it is the critical component that contributes (with CsA) to inhibiting bovine calcineurin; secondly, demonstration of a calcineurin homologue in *P. falciparum* and its susceptibility to the CsA–cyclophilin complex; and finally, demonstration of a calcineurin-dependent signalling pathway in the parasite that is inhibited by CsA in intact parasites. In contrast, we have no evidence that such a mechanism might explain the antimalarial activities of the macrolide immunosuppressant FK506, the *alter-ego* of CsA, or rapamycin. Gene amplification or cloning will probably be needed to detect FKBP-like proteins if they exist in *P. falciparum*.

In addition to its immunosuppressive and antiparasitic properties, CsA can reverse MDR in tumour cells [38]. It does so by binding to the P-glycoprotein, which is responsible for increased efflux of several anti-cancer drugs from MDR cells. The [3′-keto-MeBmt[1]]-derivatives of CsA and CsD are respectively 2- and ≥10-fold more active than CsA in reversal of MDR [32, 33]. The same compounds were, respectively, as active and 10-fold more active antimalarials than CsA, but were poorer PPIase inhibitors (Table 1) and had no effect in the calcineurin assay (Fig. 4). *P. falciparum* possesses two genes, *pfmdr1* and *pfmdr2*, encoding P-glycoprotein homologues. Originally, interest in *P. falciparum* P-glycoproteins arose from the observation that verapamil, a known P-glycoprotein-binder and reverser of tumour cell MDR, could reverse chloroquine resistance in the malaria parasite [39]. The hypothesis that chloroquine resistance is caused by a mechanism analogous to MDR is now no longer considered valid [40, 41], and cyclosporins, including [3′-keto-MeBmt[1]]-CsD, failed to reverse chloroquine resistance in *P. falciparum*.* The *pfmdr1* gene product is present in all stages of the erythrocytic cycle of *P. falciparum* and is located in the digestive vacuole membrane [42]. As mentioned above, CsA accumulates in this organelle [9]. We do not yet know whether direct inhibition of the parasite P-glycoprotein homologues would provide a basis for chemotherapy of malaria.

In summary, we have discussed two possible mechanisms for the antimalarial action of CsA: inhibition of protein folding by PPIase (cyclophilin), and inhibition of calcineurin or a similar enzyme by CsA–cyclophilin complexes. Although the first seems unlikely because of the lack of correlation between antimalarial and anti-PPIase activities, *P. falciparum* PPIase was inhibited by very low concentrations of CsA. It would be interesting to know whether the enzyme is essential in the parasite, and whether there might be less abundant PPIases that are less susceptible to CsA. We have presented evidence to support the second hypothesis for CsA and its 8′-OMe-dihydro-derivative, but it cannot explain the strong antimalarial activity of the 3′-keto-derivatives of CsA and CsD. We therefore hope to identify a parasite homologue of calcineurin and define its role in growth and development.

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