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Calcium and calmodulin antagonists inhibit human malaria parasites (*Plasmodium falciparum*): Implications for drug design

(chemotherapy/*in vitro* culture/cyclosporin A)

L. W. SCHEIBEL*†, P. M. COLOMBANI‡, A. D. HESS‡, M. AIKAWA§, C. T. ATKINSON§, AND W. K. MILHOUS¶

*Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, School of Medicine, Bethesda, MD 20814; ‡Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; §Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106; and ¶Division of Experimental Therapy, Walter Reed Army Institute of Research, Washington, DC 20307

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ABSTRACT **The malaria parasite has an obligate calcium requirement for normal intracellular growth and invasion of host erythrocytes. Calmodulin (CaM) is a vital calcium-dependent protein present in eukaryotes. We found by radioimmunoassay that free parasites contain CaM. Schizont-infected erythrocytes had CaM levels of 23.3 ± 2.7 ng per 10^6 cells compared to normals (11.2 ± 1.5 ng per 10^6 cells). CaM levels were proportional to parasite maturity. Immunoelectron microscopy identified CaM diffusely within the cytoplasm of mature parasites and at the apical end of merozoites within the ductule of rhoptries, which may explain the calcium requirement for invasion. Cyclosporin A (CsA) was also found by electron microscopic autoradiography to concentrate in the food vacuole, as do chloroquine and mefloquine, and to distribute within the cytoplasm of mature parasites. The binding of dansylated CsA to schizont-infected erythrocytes was higher than to normal erythrocytes as analyzed by flow cytometry. Kinetic analysis revealed that binding was saturable for normal and infected erythrocytes and possibly free parasites. Competition for binding existed between dansylated CsA and native CsA as well as the CaM inhibitor W-7 and the classic antimalarial chloroquine. The *in vitro* growth of *Plasmodium falciparum* was sensitive to CaM antagonists, and in large part inhibition of the parasite was proportional to known anti-CaM potency. Antagonism existed between combinations of these drugs in multi-drug-resistant strains of *P. falciparum*, suggesting possible competition for the same binding site. In addition, the malaria parasite was also susceptible to calcium antagonists.**

Ca^{2+} and Ca^{2+}-dependent metabolic activities may be affected by the classic antimalarial drugs. Ca^{2+} is an essential requisite for the growth of the malaria parasite (1), which actively accumulates Ca^{2+} (2–4). One Ca^{2+}-dependent protein, calmodulin (CaM), is critically important for many cellular metabolic activities, in particular cell growth and division (5). Drugs that interfere with CaM function by altering Ca^{2+}-dependent metabolic processes in the cell. The relative potencies of anti-CaM drugs follow a well-described structure–activity relationship to CaM. Since the classic antimalarial drugs quinacrine, quinine, and chloroquine are reported to have significant anti-CaM activity (5, 6), it is possible that the mechanism of action of the antimalarials involves anti-CaM activity in the malaria parasite. Cyclosporin A (CsA) has antimalarial activity *in vitro* and *in vivo* (7, 8). It has been shown to bind CaM, implicating CaM or other Ca^{2+}-dependent proteins in the immunosuppressive action of CsA (9, 10). CsA also reverses vincristine resistance of tumor cells, probably by a Ca^{2+}/CaM-dependent process (11).

We therefore assessed the presence and distribution of CaM within the parasite by using a radioimmunoassay and immunoelectron microscopy. Electron microscopic autoradiography and flow cytometry suggest CsA binding is presumably to parasite CaM. A study of the effects of CsA and of other CaM antagonists and the Ca^{2+}-channel blockers, alone and in combination with the classical antimalarials, was done to relate *in vitro* growth inhibition of *Plasmodium falciparum* to anti-Ca^{2+}/CaM potency and to define more closely their site of action.

METHODS AND MATERIALS

The CaM content of normal and *P. falciparum* (Colombian strain FCB_{k+}, chloroquine resistant)-infected erythrocytes was determined using a standard radioimmunoassay kit (New England Nuclear), which utilizes a high-affinity sheep anti-CaM (bovine brain) antibody, with 100% cross-reactivity to various CaM species. Immunoelectron microscopy was performed using *P. falciparum* (Brazilian strain 7G8, drug sensitive) schizonts or merozoites essentially as described by Ardeshir *et al.* (12). Electron microscopic autoradiography of *P. falciparum* (Colombian strain FCB_{k+}) exposed to ^3H photoaffinity-labeled CsA (^3H-PA-CsA) was done as described by Aikawa (13).

Flow cytometric determinations were done on *P. falciparum* (Colombian strain FCB_{k+}) schizont-infected erythrocytes obtained by gel flotation (60–80% parasitemia) and normal erythrocytes, as described for lymphocytes (9, 14). Briefly, 2×10^6 cells were incubated for 30 min at 37°C with decreasing concentrations of dansylated CsA in phosphate-buffered saline. For competitive binding studies, cells were incubated with equimolar concentrations of dansylated CsA and inhibitor [native CsA, W-7 (a CaM antagonist), and chloroquine]. Cells were then subjected to flow cytometric analysis (ultraviolet excitation wavelength, 350 nm; emission wavelength, 480–520 nm). A Becton Dickinson FACS II flow cytometer was used. Computer analysis was performed on 10,000 cells counted through the FACS, discriminating cell scatter and fluorescence.

Growth inhibition determinations were performed on *P. falciparum* strain FCB_{k+}, an Indochina clone, which is multidrug resistant (chloroquine, pyrimethamine, quinine, sulfadoxine), and on a Sierra Leone African clone, which is sensitive to these antimalarials but resistant to mefloquine. The ED_{50} (50% effective dose) for each drug was obtained by two methods using a morphologic end point as a criterion (8) and uptake of nucleic acid precursor (15).

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Abbreviations: CaM, calmodulin; CsA, cyclosporin A; ^3H-PA-CsA, tritiated and photoaffinity-labeled CsA; FIC, fractional inhibitory concentration.

†To whom reprint requests should be addressed.

Drug–drug interactions between CaM inhibitors were done using a semiautomated microdilution technique as described by Martin *et al.* (15). Computer-generated concentration–response curves were analyzed by nonlinear regression and 50% inhibitory concentrations were calculated (IC_{50}) for each drug, both alone and in combination. Fractional inhibitory concentrations (FIC) were calculated (16). The FIC index is simply a mathematical representation of whether the FIC of one drug is reduced, unchanged, or increased in the presence of the second drug. A FIC index of 1.0 would represent additivity or independence, whereas indices >1.0 would indicate antagonism and indices <1.0 would indicate potentiation or synergism.

RESULTS

CaM Content and Distribution in *P. falciparum*. Previous studies demonstrated that Ca^{2+} is essential to the growth and development of *P. falciparum* (1) and that CsA, a CaM antagonist, exhibits *in vitro* and *in vivo* antimalarial activity (7, 8). We therefore first determined the presence of CaM, a vital Ca^{2+}-dependent protein, in erythrocytes infected with *P. falciparum*. Normal erythrocytes, rings, and schizont-infected erythrocytes were analyzed for CaM content. Normal erythrocytes contained the lowest concentration of CaM [11.2 ± 1.5 (SEM) ng per 10^6 cells], the ring (17.8 ± 1.1) and the schizont (23.3 ± 2.7) stages of infected erythrocytes differed in CaM content ($P < 0.05$), and both contained a significantly greater level of CaM than did normal erythrocytes ($P < 0.001$). In other studies, free parasites contained 41 ng per 10^6 parasites.

To determine the localization of CaM in the parasite, we performed immunoelectron microscopy using a LR White resin technique with anti-CaM antibody and protein A-gold. Gold particles identified the location of CaM within the parasites. In the trophozoites and schizonts (Fig. 1*A*), gold label was found scattered through the cytoplasm, indicating a diffuse pattern of CaM in these mature parasites. By contrast, in the merozoite, the CaM was concentrated in the apical complex (Fig. 1*B*).

Ultrastructural Analysis of Binding. Previous studies in our laboratory showed that CsA is rapidly internalized into the cytoplasm of T lymphocytes and binds to a number of receptors, including CaM (9, 17). An effort to better localize receptors within the malaria parasite prompted a series of experiments using a derivative of CsA (^{3}H-PA-CsA), which was labeled with both ^{3}H and a photoaffinity group (17). When exposed to ultraviolet light, the photoactive group on this derivative covalently binds to its adjacent protein receptor. Parasitized erythrocytes were incubated with this derivative, exposed to UV light, and then processed for electron microscopy autoradiography. Fig. 2 is an electron photomicrograph of mature parasites contained within erythrocytes.

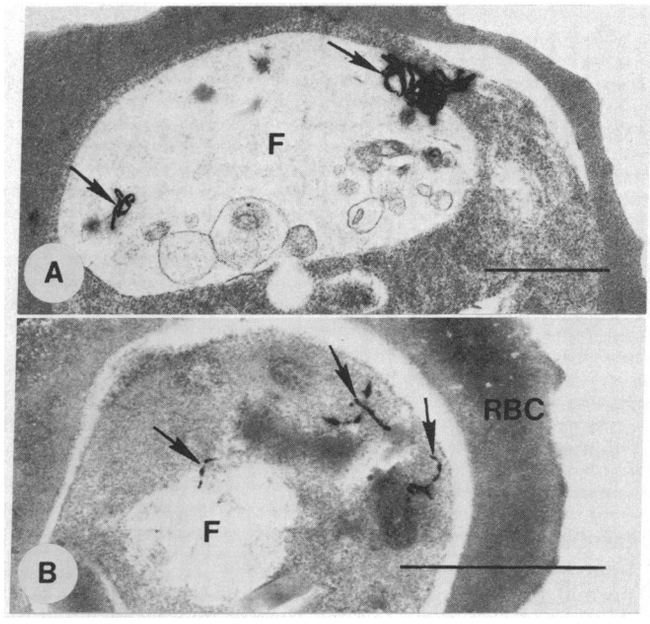

FIG. 2. Electron micrograph autoradiographs. (*A*) Section of mature *P. falciparum* (Colombian strain FCB_{k+}, chloroquine resistant) exposed to ^{3}H-PA-CsA for 2 weeks. Silver grains (arrows), which indicate the concentration site of CsA, are present within a swollen food vacuole (F). (Bar = 1 μm.) (*B*) Section of *P. falciparum* trophozoite exposed to ^{3}H-PA-CsA for 8 months. Silver grains (arrows) are associated with the food vacuole (F), as well as the parasite cytoplasm. Silver grains are not located over the host erythrocyte (RBC) cytoplasm. (Bar = 1 μm.)

After relatively short exposure times (2–3 weeks), silver grains were located primarily over the food vacuole of the parasite (Fig. 2*A*). This distribution pattern was similar to that exhibited by chloroquine and mefloquine when analyzed by similar techniques (13, 18). Long exposure times (8 months) revealed a more diffuse pattern in the cytoplasm of the parasite (Fig. 2*B*). Few silver grains were associated with the erythrocyte cytoplasm. Additional lesions seen include loss of ribosomes, dilatation of the endoplasmic reticulum, and degeneration of the nucleus.

Flow Cytometric Analysis. Initial fluorescent microscopic studies using a fluorescent dansylated derivative of CsA showed that all stages of the malaria parasite concentrated the drug, whereas normal uninfected erythrocytes bound comparatively little dansylated CsA. Therefore, quantitative analysis of binding to normal and infected erythrocytes was performed using flow cytometry. Previous studies with T lymphocytes showed this technique to be a reliable method to analyze the binding kinetics of CsA (9). Fig. 3 depicts a

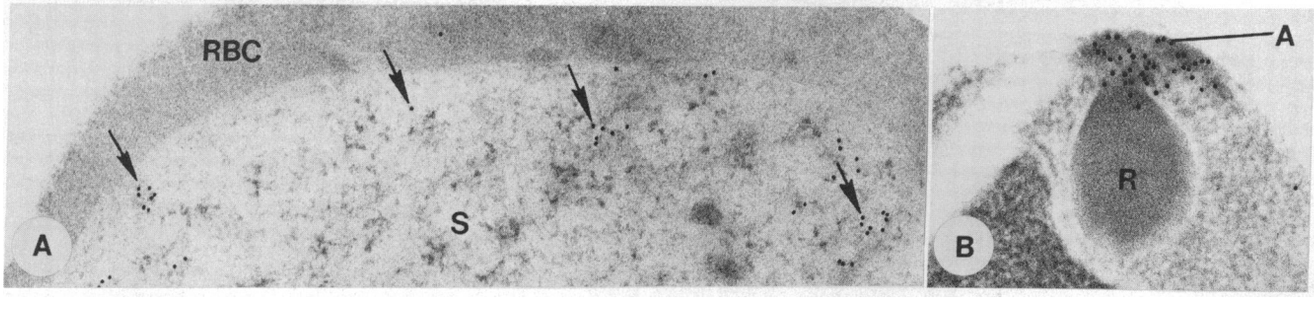

FIG. 1. Immunoelectron micrographs. (*A*) Section of an early schizont of *P. falciparum* (Brazilian strain 7G8, drug sensitive) incubated with anti-CaM antibody and protein A-gold. Gold particles (arrows) are scattered diffusely over the schizont (S) cytoplasm. Little label is associated with the host erythrocyte (RBC). (×43,000.) (*B*) Section of merozoite. Gold particles indicate the location of CaM at the apical end (A) and the ductule of rhoptries (R). (×52,000.)

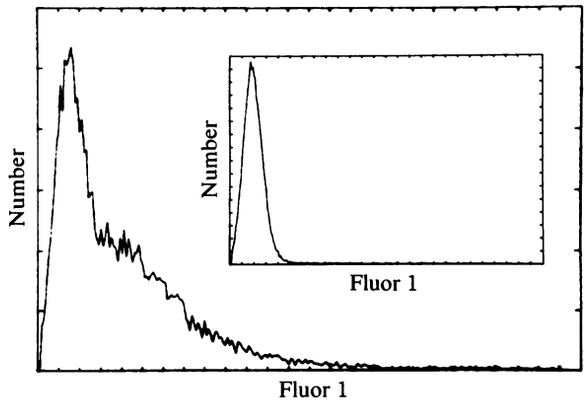

FIG. 3. Two-dimensional histograms plotting number of normal (*Inset*) or schizont-infected erythrocytes versus fluorescence (arbitrary units).

two-dimensional histogram plotting cell number vs. mean cell fluorescence obtained by flow cytometry after incubating normal (*Inset*) and schizont-infected erythrocytes with dansylated CsA [note the low level of fluorescence and the uniform population of normal erythrocytes (mean fluorescence = 16)]. For schizont-infected erythrocytes, two peaks of fluorescence were observed in all experiments, which discriminated two populations of cells, one with a mean fluorescence of 15, the other with a mean fluorescence of 48. When separated by sorting techniques on the flow cytometer, the low fluorescence population was made up of normal erythrocytes contaminating the schizont-infected cell population. The population of sorted cells with higher fluorescence contained >95% schizont-infected erythrocytes. Mean fluorescence of the infected erythrocytes was 40–150% above controls in separate experiments.

Using increasing concentrations of dansylated CsA, binding curves were generated for both the normal and parasitized erythrocytes. Fig. 4 presents the binding curves generated by plotting mean fluorescence vs. CsA concentration. These binding curves showed that there was increased binding of CsA with increasing concentration of the dansylated CsA in the incubating media. Binding to normal erythrocytes was clearly saturable with half-maximal binding at 0.3 μM (apparent K_d). Binding to parasitized erythrocytes appeared saturable with a similar K_d.

In separate studies, the specificity of binding was assessed by analyzing the inhibition of dansylated CsA binding to infected erythrocytes and to free parasites by unmodified CsA as well as the CaM inhibitor W-7 and chloroquine. Infected erythrocytes and free parasites were incubated in dansylated CsA alone or with an equimolar concentration of

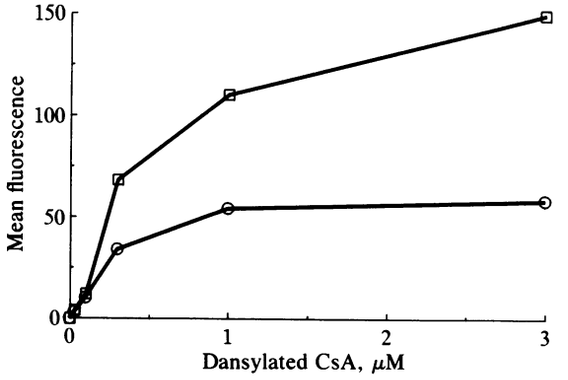

FIG. 4. Plot of mean fluorescence versus concentration of dansylated CsA for normal (○) and parasitized erythrocytes (□).

the above drugs (since higher concentrations resulted in cell lysis), and mean cell fluorescence was compared. Infected erythrocytes demonstrated an ≈20% decrease in mean fluorescence when incubated simultaneously with native CsA or W-7 but not with chloroquine. Free parasites, however, showed a 40% decrease in cell fluorescence when incubated with native CsA, W-7, or chloroquine. These studies suggested that a significant proportion of CsA binding was specific and that W-7 and chloroquine competed with CsA for binding.

Growth Inhibition of *P. falciparum* by CaM Inhibitors and Calcium Antagonists. A continuous culture of *P. falciparum* was used to analyze the inhibitory potential of CsA and other classes of Ca^{2+}/CaM antagonists in relation to the effect of classical antimalarials (Table 1). These drugs exhibited graded antimalarial activity, reflecting differing anti-CaM activity based on reported structural prerequisites for CaM binding (19). Known CaM antagonists, CsA, compound R24571 (a derivative of the antimycotic agent miconazole), and W-7, a structural analog of smooth muscle relaxing agents, inhibited *P. falciparum in vitro*. The phenothiazines also reflected their anti-CaM activity with trifluoperazine being more effective than chlorpromazine in antiplasmodial activity. Both phenothiazines were more effective than the butyrophenone haloperidol. The local anesthetics were 10–100 times less potent than the phenothiazines, reflecting their relatively poor anti-CaM activity (20). The clinically useful antimalarial agents, in general, obey the structural prerequisites cited as important for anti-CaM activity. They also possess local anesthetic properties (20). Their rank order of antimalarial activity within their class reflected their reported anti-CaM effect. In comparison to the other anti-CaM agents, however, these drugs were much more potent inhibitors of *in vitro* growth of *P. falciparum*. The malaria parasite is sensitive to changes in the availability of cations. Extracellular chelators are capable of interacting with extracellular cations, thereby inhibiting the *in vitro* growth of *P. falciparum* (1, 14). We therefore analyzed the ability of Ca^{2+} channel blockers to

Table 1. Growth inhibition by Ca^{2+}/CaM agents

Agent	ED_{50}, μM
CaM antagonists	
CsA	0.65
R24571	2.3
W-7	2.1
Phenothiazines	
Chlorpromazine	4.5
Trifluoperazine	1.9
Butyrophenone	
Haloperidol	8.8
Local anesthetics	
Dibucaine	15.0
Tetracaine	186.0
Propranolol	6.4
Antimalarials	
Quinine	0.24
Quinadine	0.066
Mepacrine (quinacrine)	0.028
Chloroquine	0.31
Calcium antagonists	
Perhexiline maleate	8.1
Verapamil hydrochloride	7.7
Diltiazem hydrochloride	13.0
Nifedipine	92.0

ED_{50}, concentration required to reduce *in vitro* growth of *P. falciparum* (strain FCB_{K^+}, chloroquine resistant) 50% after exposure for 3 days. Calcium antagonists were from R. Goldstein (Uniformed Services University of the Health Sciences), quinine was from Aldrich, and remaining agents were from Sigma.

inhibit growth of *P. falciparum*. The CaM inhibitors CsA, compound R24571, and W-7 were more effective in inhibiting the growth of *P. falciparum* than were the Ca^{2+}-channel blockers perhexiline, verapamil, diltiazem, and nifedipine (Table 1). Verapamil was more effective than the other drugs tested, which may be attributed to both its Ca^{2+} antagonism and to a direct effect on CaM (5, 21, 22).

Drug–Drug Interactions of CaM Inhibitors on the Growth of *P. falciparum*. Since these drugs directly inhibit the growth of the parasite according to their anti-CaM potency, one drug should alter the *in vitro* antimalarial potency of the other. Using modifications of the semiautomated microdilution technique (15), a series of experiments was performed in which concentrations of these drugs alone and in varying concentrations with each other were added to two clones of *P. falciparum*, of which one was multidrug resistant and the other was a sensitive clone. FIC indices were tabulated in Table 2. The results demonstrated marked antagonism between pairs of classic CaM antagonists (CsA, R24571, W-7, and chlorpromazine). Since known antimalarials such as mepacrine (quinacrine), quinine, and chloroquine (5, 6) have been reported to inhibit CaM, the activities of these drugs were also assessed in combination with CsA, R24571, and W-7. Significant antagonism existed between many of the drug pairs, especially in the multidrug-resistant clone.

DISCUSSION

Previous studies indicated that Ca^{2+} is important to the invasion, growth, and development of the malaria parasite *in vitro* (1) and that extracellular chelators inhibit parasitic growth (1, 14). Ca^{2+}-dependent metabolism can be inhibited at other levels: at the membrane level with Ca^{2+}-channel blockers and at the level of Ca^{2+}-dependent proteins with CaM antagonists. The antagonists initially shown to inhibit CaM have subsequently been shown to inhibit variably an entire class of Ca^{2+}-dependent proteins in mammalian cells (5). CsA, a noncytotoxic immunosuppressive drug, has antimalarial effects and appears to inhibit CaM and phospholipase A2, two Ca^{2+}-dependent proteins. In addition, CsA reverses vincristine resistance in a T-cell leukemia line (11), a property associated with CaM inhibitors and/or Ca^{2+}-channel blockers. The binding of CsA to CaM and other Ca^{2+}-dependent proteins may effectively block Ca^{2+}-dependent cellular activities in the growing parasite. This mechanism may be shared by mepacrine, quinine, and chloroquine, since they also are reported CaM antagonists (5, 6).

Using a CaM radioimmunoassay we showed that parasitized erythrocytes have increased CaM levels with increasing parasite maturity. Experiments using electron microscopy and gold-labeling of an anti-CaM antibody, demonstrated CaM is concentrated in the apical complex of the merozoite (Fig. 1*B*), which is believed to be important in the invasion of the host erythrocyte. These data suggest that erythrocyte penetration is a Ca^{2+}/CaM-dependent process. Drugs such as CsA may act on CaM at the apical end of the merozoite, thereby inhibiting Ca^{2+}/CaM activity and preventing invasion of the erythrocyte (7). Using a photoaffinity derivative of CsA and electron microscopy, we also demonstrated uptake of CsA within the cytoplasm of the mature parasite with a distribution similar to the CaM gold-labeling. In other sections there is a concentration of CsA within the food vaçuole, which has also been observed with chloroquine (reported to be a CaM inhibitor).

Table 2. Fractional inhibitory concentrations of drug combination studies

	Indochina clone (multidrug resistant)			Sierra Leone clone (multidrug sensitive)		
	CsA	R24571	W-7	CsA	R24571	W-7
R24571	1.49	—	2.38	1.13	—	1.31
W-7	2.27	2.38	—	1.26	1.31	—
Chlorpromazine	1.11	1.39	1.25	1.63	1.16	1.10
Mepacrine	1.19	2.23	1.06	1.09	1.21	1.21
Quinine	1.20	2.68	3.43	0.83	1.66	2.10
Chloroquine	1.89	3.28	0.91	1.25	1.25	1.35
Verapamil	0.86	4.64	1.83	0.80	0.93	1.18
Diltiazem	1.27	1.50	1.43	1.12	1.26	1.16

Earlier experiments using fluorescent microscopy showed that dansylated CsA was rapidly partitioned selectively into the parasite within *P. falciparum*-infected erythrocytes. This was corroborated by flow cytometric techniques, which documented that the parasitized erythrocytes possessed at least a 40% increase in cell fluorescence, compared to uninfected erythrocytes. Flow cytometric techniques also demonstrated that binding of the fluorescent CsA appeared saturable with an apparent K_d of 0.3 μM, which correlates closely with the K_d of CsA binding to intact T lymphocytes and CaM. A high level of specific binding was suggested by a 40% decrease in binding with equimolar concentrations of native CsA, W-7, or chloroquine, suggesting the antimalarials may interact with other anti-CaM agents in the malaria parasite.

CsA, classic CaM antagonists, and Ca^{2+}-channel blockers all effectively inhibited growth of *P. falciparum*. The correlation between the relative potency of the *in vitro* antiplasmodial effect and reported anti-CaM potency of these drugs suggests a possible role for CaM in parasite cell functions. Potency reportedly depends on specific hydrophobic and polar regions common to the CaM inhibitors (19). In addition, these drugs have effects on other Ca^{2+}-dependent proteins and possibly other nonspecific effects. The classical antimalarial agents as a class were much more effective inhibitors than indicated by their anti-CaM potential. The rank order of their antiplasmodial activity, however, reflected their anti-CaM activity. It was this discrepancy that prompted our drug–drug interaction studies.

Presumably, anti-CaM inhibitors act by binding to hydrophobic regions on the CaM molecule, which are exposed by a Ca^{2+}-induced conformational change thereby preventing activation of secondary Ca^{2+}-dependent enzymes—e.g., cyclic nucleotide phosphodiesterase, adenylate cyclase, protein kinases, etc. (23). If these drugs have the same site of activity in the malaria parasite they should antagonize one another. Our results on the *in vitro* growth of *P. falciparum* showed marked antagonism between the CaM antagonists CsA, R24571, and W-7, as well as varying degrees of antagonism between these drugs and the Ca^{2+}-channel blockers or the classical antimalarials. This antagonism was more readily demonstrated in the multidrug-resistant strain of plasmodia (Indochina clone). Such antagonism suggests competition for the same receptor binding site. The variation between FIC values >1.0 suggests that the degree of antagonism may depend on differing binding affinities or rates of uptake in the multicompartment parasite/erythrocyte system. Studies by Martin *et al.* (15) showed potentiation of chloroquine by verapamil, which is compatible with sequential actions on Ca^{2+} flux and CaM or on CaM and secondary proteins that bind CaM (23). Ca^{2+} antagonists thus appear to be multifunctional, whereby a block of Ca^{2+} uptake (verapamil and nifedipine), or mobilization of intracellular Ca^{2+} (diltiazem), will mimic the Ca^{2+} deficiency state created by extracellular chelators. In addition, certain Ca^{2+}-channel blockers (verapamil) bind to and antagonize CaM. Conversely, some CaM antagonists may also exhibit Ca^{2+}-channel blocking activities (5, 24).

An understanding of the regulation of Ca^{2+} and the role of CaM in the malaria parasite may partially explain both the

actions of the classic antimalarial drugs quinacrine, quinine, and chloroquine (5, 6) and the suppressive activity of CsA on rodent and human malaria *in vitro* and *in vivo* (7, 8). In addition, the well known immunomodulating effect of chloroquine may be a result of its anti-CaM potential, similar to the CsA effect on Ca^{2+}/CaM functions of T lymphocytes. While much larger doses of chloroquine are required for treatment of collagen vascular diseases, antimalarial doses are reported to exert rather profound effects on the immune system, especially T-cell-dependent responses (see ref. 25).

Drug interaction with Ca^{2+} metabolism and functions mediated by Ca^{2+}-dependent proteins may explain the activity of a number of drugs on other parasites. The parasitic roundworm *Ascaris* and three species of African trypanosomes have been shown to possess CaM (26, 27). The phenothiazines have both weak anthelmintic properties and markedly inhibit trypanosome growth with disintegration of pellicular microtubules (28). The benzimidazoles, a class of broad spectrum anthelmintics, also cause disruption of microtubules. The action of praziquantel, another important anthelmintic, is related to Ca^{2+}. α-Difluoromethylornithine inhibits polyamine synthesis and has marked antitrypanosomal activity. It is of interest that Ca^{2+}/CaM mediates DNA and polyamine synthesis as well as microtubular assembly/disassembly, both of which are necessary for cell growth and division (23). Our understanding of all these mechanisms in parasites is presently incomplete and warrants further investigation.

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