Calmodulin, Cyclophilin, and Cyclosporin A

P. M. Colombani *et al.* (*1*) suggest that binding of cyclosporin to calmodulin may account for the immunosuppressive effects of cyclosporin A (CsA). They further suggest that cyclophilin, a specific cytosolic binding protein for CsA (*2*), may be structurally related to calmodulin or copurify with it. Because, as outlined by Colombani *et al.*, calmodulin would be an attractive target for the action of cyclosporin, we have done additional experiments, the results of which do not support the concept that CsA is an inhibitor of calmodulin or that cyclophilin and calmodulin share similar properties.

The evidence Colombani *et al.* present in support of their hypothesis that CsA exerts its biological action through the antagonism of calmodulin includes data showing the binding of CsA to calmodulin and the inhibition of calmodulin-activated phosphodiesterase. Figure 1 in their report shows that the fluorescence emission of dansylated calmodulin was enhanced by the binding of CsA as well as by the binding of the calmodulin inhibitors calmidazolium (R24571) and W7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide]. However, there are at least two types of interactions between a drug and calmodulin: (i) a calcium-dependent, saturable, high-affinity interaction and (ii) a calcium-independent, nonsaturable, low-affinity interaction (*3*). Therefore, the demonstration of calcium dependence and a kinetic analysis of the interaction between CsA and calmodulin are essential. In their kinetic analysis (their figure 2), Colombani *et al.* show a direct linear relationship between the concentration of dansyl calmodulin and the apparent dissociation constant. Since the dissociation constant should be independent of the concentration of receptor (in this case calmodulin), these data are most consistent with a nonsaturable, nonspecific binding of CsA to calmodulin, reminiscent of the kinetics observed between the interaction of other hydrophobic drugs with the molecule (*3, 4*). Furthermore, Colombani *et al.* do not address the calcium dependence of the binding of CsA to calmodulin.

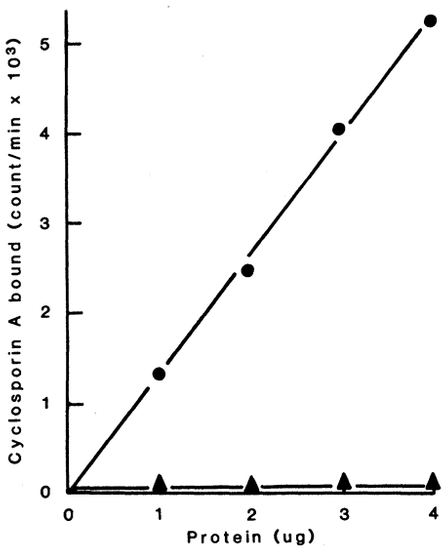

Fig. 1. Comparison of CsA binding by cyclophilin and calmodulin. Equivalent concentrations of homogenous bovine cyclophilin and bovine calmodulin (Calbiochem) were tested for CsA binding by Sephadex LH-20 column assay (*2*). Protein solutions (100 μg/ml) were prepared in calcium buffer (50 μ*M* glycyl-glycine, 25 m*M* CH_3COONH_4, 3 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, 5 m*M* dithiothreitol, *p*H 8.0), and assays were performed with LH-20 columns preequilibrated with calcium buffer. Results show [^{3}H]CsA binding proportional to the concentration of cyclophilin (●), but no binding at any concentration of calmodulin (▲).

Colombani *et al.* also examined the binding of dansyl-CsA to bovine calmodulin and calf thymus cyclophilin and report a similar increase in fluorescence emission. What is not discussed is that the dansyl group [5-(dimethylamino)-1-naphthalenesulfonyl-R] is structurally related to the napthalenesulfonamide calmodulin inhibitors, and another dansylated protein, dansyl-cadavarine, is an effective calmodulin inhibitor (*5*). Therefore, the interaction of the dansyl moiety of the CsA derivative with calmodulin may account for the enhanced fluorescence emission. We present evidence that native calmodulin does not bind native CsA in a minicolumn assay (Fig. 1), nor does it compete with the binding of CsA to cyclophilin. These experiments were conducted in the presence and absence of calcium and under conditions that approximate those used by Colombani *et al.*

Colombani *et al.* also suggest that a competitive relationship exists between the binding of dansyl-CsA and the two calmodulin inhibitors to T lymphocytes. However, a Lineweaver-Burk plot of competition between dansyl-CsA and the calmodulin inhibitors for binding sites on or in T lymphocytes would only indicate competition if an intercept occurs significantly above the origin. Their figure 3 indicates that all four lines intercept the *y*-axis at the origin, which is not an acceptable criterion for competition.

Colombani *et al.* also report 50% inhibition of calcium-calmodulin–dependent activation of phosphodiesterase by 100 n*M* of CsA in the presence of 0.5 unit of calmodulin (their figure 4), a strong indication of a biological target of action for CsA. Using similar assay conditions, we were unable to demonstrate inhibition of calmodulin-activated phosphodiesterase by CsA at concentrations up to 20 μ*M*, whereas significant inhibition by the calmodulin inhibitor trifluoperazine was noted (Fig. 2).

Colombani *et al.* raise the possibility that cyclophilin may be calmodulin or a closely related calcium-binding protein because of cross-reactivity in a calmodulin radioimmunoassay. However, the experimental conditions of their assay have not been made available, and the specificity of the polyclonal antiserum is not defined. As indicated, a shared antigenic determinant or a minor contaminant in the cyclophilin preparation may have contributed to cross-reactivity. We examined serum samples (four human and five rabbit) that contained antibodies (IgG) to cyclophilin and did not observe cross-reactivity with calmodulin.

Colombani *et al.* suggest that, despite the lack of sequence homology in the first five to ten amino acids, cyclophilin (M_r, 17,628; *p*I 9.6) may be a structural analog of calmodulin (M_r, 16,680; *p*I 3.9). However, the complete sequence of cyclophilin has been determined (*6*), and there is no evidence of homology with calmodulin or any other

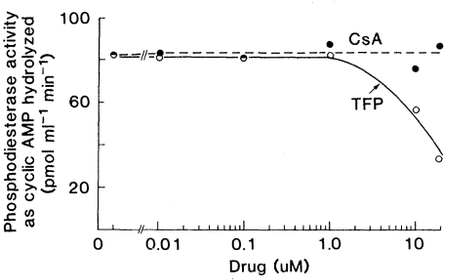

Fig. 2. Effect of cyclosporin A (CsA) and trifluoperazine (TFP) on the activity of calmodulin-stimulated cyclic nucleotide phosphodiesterase. Phosphodiesterase was prepared from rat cerebrum and assayed in the presence of 0.5 unit of calmodulin (Calbiochem) (*7*). One unit of calmodulin is defined as the concentration of calmodulin required to produce 50% of the maximum (fourfold) activation of phosphodiesterase. TFP was dissolved in water and CsA in ethanol, and the effect of increasing concentrations of drugs and their vehicles on the activation of phosphodiesterase was determined. The results demonstrate no inhibition of calmodulin-activated phosphodiesterase by CsA under conditions where expected inhibition by TFP occurred.

calcium-binding protein, even when a potential evolutionary relationship is considered.

In other experiments designed to determine whether or not cyclophilin and calmodulin shared similar properties, we found that cyclophilin could not activate calmodulin-sensitive phosphodiesterase or bind the calmodulin inhibitor trifluoperazine.

In summary, we were not able to demonstrate the critical biological effect of CsA on calmodulin activation of phosphodiesterase reported by Colombani *et al.* We present other evidence that structurally and functionally distinguishes calmodulin from cyclophilin. It is possible that cyclosporin may interrupt a regulatory or metabolic pathway that is in some way modulated by calcium and calmodulin. However, the extreme hydrophobic properties of CsA and the well-defined hydrophobic domain of calmodulin may account for much of the experimental data presented by Colombani *et al.*

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Response: In our initial report (*1*), we hypothesized that the effect of cyclosporin (CsA) on T-lymphocyte activation (which appears to be calcium-dependent) may be at the level of inhibition of calmodulin or other calcium-dependent proteins. We also attempted to correlate our findings with those of Handschumacher *et al.*, who have suggested that cyclophilin is the specific cytoplasmic receptor for CsA (*2*). Hait *et al.*, using a Sephadex LH-20 column assay to analyze the interaction of CsA and cyclophilin, do not demonstrate a specific interaction of CsA with calmodulin. They therefore suggest that this interaction is nonspecific.

We were perplexed by the inability of the LH-20 column to demonstrate binding between CsA and calmodulin, although Hiestand has shown (*3*) that CsA binds to calmodulin when increased concentrations of [^{3}H]CsA are used in the column assay (*3*). The LH-20 column provides a hydrophobic gel bed that weakly binds and retards elution of hydrophobic small compounds such as CsA. These compounds, upon binding to a receptor protein, will elute in the void volume as a complex. This principle requires that the binding protein be excluded from the gel or not significantly interact with the gel bed. We therefore analyzed the binding of CsA to calmodulin using the LH-20 column assay. We found that calmodulin (M_r, 16,700) significantly interacted with the LH-20 column and did not elute in the void volume (despite its molecular weight), in contrast to chymotrypsinogen (M_r, 25,000), and ribonuclease A (M_r, 13,700), both of which eluted in the void volume with blue dextran on the LH-20 column. The elution of calmodulin was similar to that of CsA. This hydrophobic column interaction of calmodulin would provide the simplest explanation for the fact that Hait *et al.* did not demonstrate CsA binding to calmodulin with their assay. This interaction of calmodulin with the LH-20 column is not altogether surprising because calmodulin has two different hydrophobic sites that prevent the formation of the CsA-calmodulin complex.

Because the fluorometric demonstration of CsA binding to calmodulin in our original report (*1*) was of concern, we analyzed CsA binding to calmodulin in a nonhydrophobic column of Sephadex G-25, as described by other investigators (*4*). We found that calmodulin elutes with blue dextran in the void volume of this column in the absence or presence of calcium, while CsA does not, as expected because of the differences in molecular weight. Using this column, we demonstrated that there was significant binding of CsA to calmodulin and that the binding was calcium-dependent (Fig. 1).

The data presented in our original report demonstrated that the binding of CsA to calmodulin is consistent with a high affinity association in the micromolar range, which is saturable, rather than in the low affinity (millimolar range) association, which is nonsaturable [figure 1 in (*1*)]. Our kinetic analysis demonstrated that, as in the case of the calmodulin inhibitor, calmidazolium (R24571), the absolute increase in fluorescence of dansylated calmodulin upon CsA binding is influenced by the concentration of dansylated calmodulin [figure 2 in (*1*)]. Therefore a single binding curve based on a

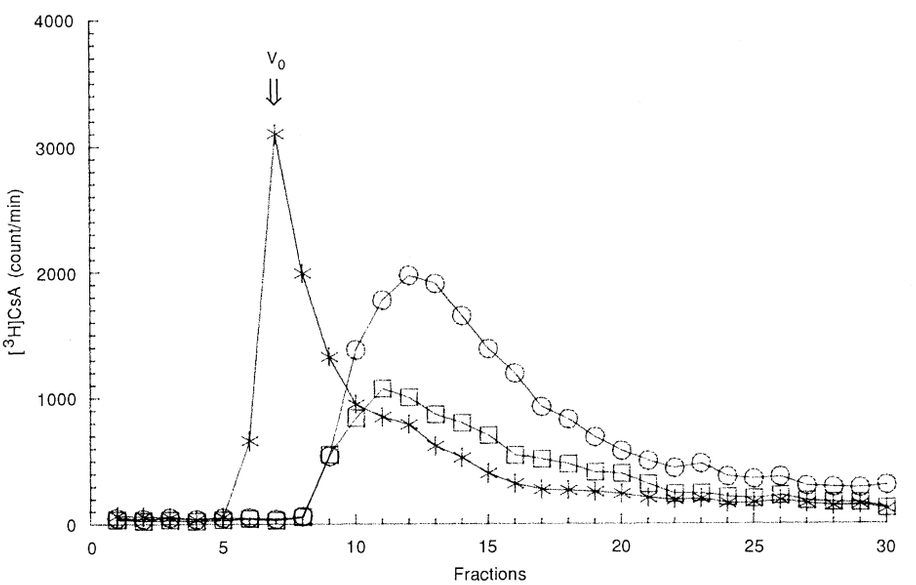

Fig. 1. Elution pattern of [^{3}H]CsA on a Sephadex G-25 column alone or with calmodulin. A gel-filtration column of fine Sephadex G-25 was prepared in a column 1.0 × 10 cm. Sephadex G-25 fine granules were washed for 3 hours in glass-distilled water, columns poured to a bed volume of 4.3 ml (1.0 × 5.5 cm) and washed with several bed volumes of appropriate buffer solutions. The buffer solution contained 20 m*M* tris-chloride with or without 0.1 m*M* $CaCl_2$ and 3 m*M* $MgCl_2$, *p*H 7.5. Column filtration was performed at 23°C. Samples (200 μl) were added to the column and eluted by gravity. The void volume (V_0) of the column was calculated from the elution volume of blue dextran 2000 and corresponded to collection fraction 8 (2.2 ml). The elution volume of calmodulin in the presence or absence of Ca^{2+} in the buffer solution corresponded with the calculated void volume of the column. Less than 10% of [^{3}H]CsA alone (○) eluted in the void volume of this column. When incubated with calmodulin (200 μg) in the absence of Ca^{2+} and Mg^{2+}, the [^{3}H]CsA elution profile was basically unchanged (□). When incubated with calmodulin in the presence of Ca^{2+} and Mg^{2+} (*), the [^{3}H]CsA elution profile shifted to the same profile as that of calmodulin, that is, peak counts within the void volume.