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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REFERENCES

1. P. M. Colombani, A. Robb, A. D. Hess, *Science* **228**, 337 (1985).
2. R. E. Handschumacher, M. W. Harding, J. Rice, R. J. Drugge, D. W. Speicher, *ibid.* **226**, 544 (1984).
3. R. M. Levin and B. Weiss, *Mol. Pharmacol.* **13**, 690 (1977).
4. ———, *J. Pharmacol. Exp. Ther.* **208**, 454 (1979).
5. S. Anders, K. Sandvig, S. Olsnes, *Biochem. Biophys. Res. Commun.* **117**, 562 (1983).
6. M. W. Harding, R. E. Handschumacher, D. W. Speicher, *J. Biol. Chem.*, in press.
7. G. Lee and W. N. Hait, *Life Sci.* **36**, 347 (1984).

7 October 1985; accepted 3 March 1986

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

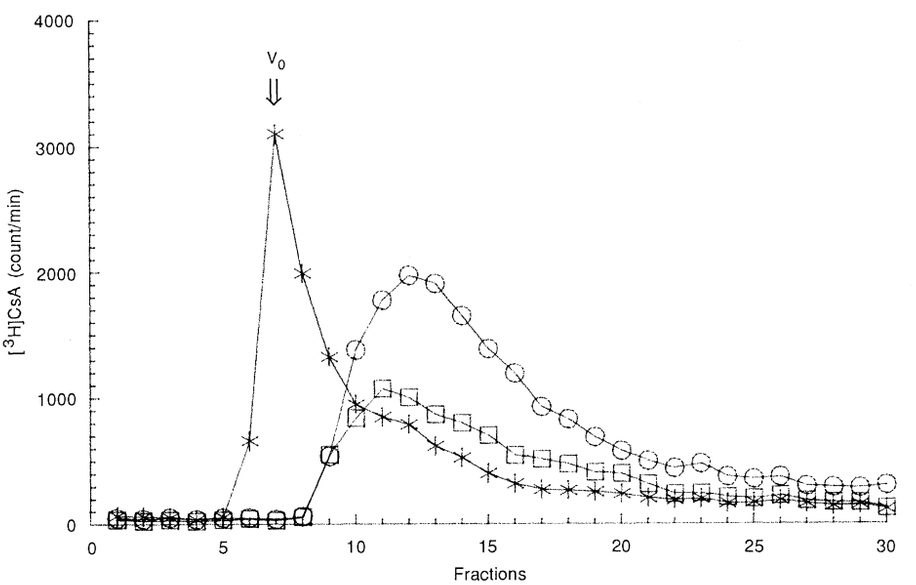

Fig. 1. Elution pattern of [^{3}H]CsA on a Sephadex G-25 column alone or with calmodulin. A gel-filtration column of fine Sephadex G-25 was prepared in a column 1.0 × 10 cm. Sephadex G-25 fine granules were washed for 3 hours in glass-distilled water, columns poured to a bed volume of 4.3 ml (1.0 × 5.5 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.

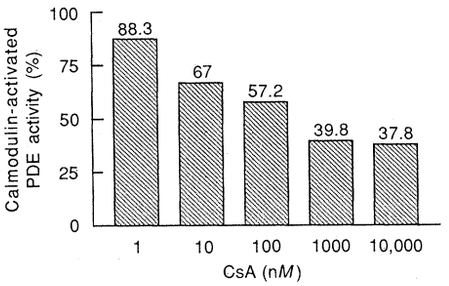

Fig. 2. Inhibition of calmodulin-activated PDE activity with cyclosporin. Ability of calmodulin to activate phosphodiesterase [measured as absorbance at 660 nm (A_{660})] in the presence of increasing concentrations of CsA was assessed as described (*1*). Data are expressed relative to calmodulin-activated PDE alone (100% positive control) and to the baseline A_{660} of PDE alone (0% negative control) in the presence of CsA. SE from six experiments varied from 5.3 to 21.1%.

single dansylated calmodulin concentration would be an inaccurate estimation of the dissociation constant. The technique we used would eliminate the error of these apparent dissociation constants (*5*). CsA-calmodulin binding requires calcium [Fig. 1 and fluorimetric data (unpublished)]. Binding of CsA to intact peripheral blood lymphocytes is also calcium-dependent and can be significantly altered by modifying intracellular calcium with EGTA or with calcium ionophores (*6*). Our finding that binding is saturable and calcium-dependent is inconsistent with a nonspecific interaction.

In our graphic depiction of the competitive inhibition of binding of dansylated CsA to intact lymphocytes by native CsA and calmodulin inhibitors, we primarily wished to demonstrate the same *y*-intercept for each line having different slopes indicating *competitive* rather than noncompetitive inhibition of binding. The lines do not pass through the origin because binding in this system is saturable.

With our assay, we observed an inhibition of calmodulin's ability to activate 3′,5′-cyclic-nucleotide phosphodiesterase (PDE). To do a PDE assay in this system, it is necessary to limit the PDE and calmodulin concentrations, because the affinity for calmodulin to PDE is high (in the nanomolar range). Reaction conditions are highly variable because of the extreme hydrophobic nature of CsA. Figure 2 depicts a summary of six dose-response experiments showing inhibition of calmodulin by CsA, and Fig. 3 shows a typical time course of this inhibition. Similarly, R24571, which is also highly hydrophobic, variably inhibits calmodulin activity depending on *p*H, temperature, and buffer solutions or concentrations of the various reactants, or both. Trifluoroperazine, however, is much more water-soluble and a more reliable inhibitor of calmodulin in vitro.

Using a commercially available polyclonal antibody to calmodulin (Amersham), we found that cyclophilin cross-reacted in a radioimmunoassay for calmodulin. We hypothesized that calmodulin and cyclophilin might be related proteins. Hait *et al.* have sequenced cyclophilin, found no homology to calmodulin, and have developed antibodies to cyclophilin that do not cross-react with calmodulin. We must assume that the polyclonal antisera we used had recognized a common epitope to the tertiary structures of both calmodulin and cyclophilin, although their primary structures are different. The assay in which antibodies to cyclophilin were used to assess immunologic cross-reactivity was not specified by Hait *et al.* and may help explain their findings. Immunologic cross-reactivity at one shared epitope cannot be determined by immunoprecipitation or immunodiffusion techniques because lattice formation cannot occur. Such cross-reactivity can best be documented in a competitive-inhibition assay such as the radioimmunoassay used in our original studies. In any case, it appears that more sophisticated techniques, such as Western blotting, should be used to study the immunological relation (receptor site similarities) between calmodulin and cyclophilin. Along similar lines, we used an affinity labeled CsA derivative that covalently binds to its receptor after ultraviolet irradiation. We found that CsA binds four to five distinct cytoplasmic proteins, with molecular weights of approximately 20,000 or less and that three of the five cross-react with the polyclonal calmodulin antisera. These data suggest that there is more than one receptor for CsA, one of which is calmodulin (*7*).

Other calmodulin inhibitors will competitively inhibit CsA binding and operationally substitute for or work synergistically with CsA in mediating immunosuppression (*8*). Use of the LH-20 column assay to analyze CsA binding to cytoplasmic proteins must be approached cautiously with attention to assay conditions and to the possibility that nonspecific interaction of proteins with the column can yield false negative results.

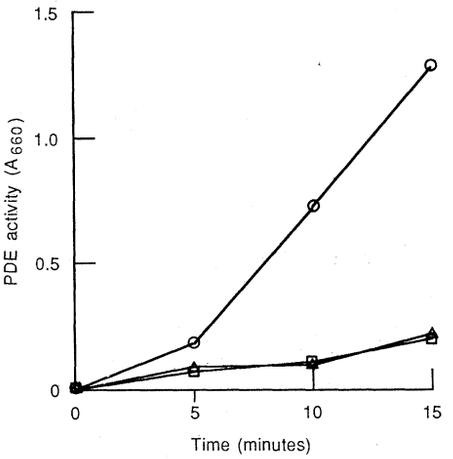

Fig. 3. PDE activity. PDE (0.5 U) was preincubated with CsA (□), with 5 U calmodulin (○) or 5 U calmodulin and $3 \times 10^{-6}M$ CsA (△) in tris buffer solution at 30° with 5′-nucleotidase (1 mg/ml) for 15 minutes in a 0.3-ml reaction volume. One unit of calmodulin will stimulate 0.016 activated units of PDE to 50% maximum activity. Reactions were initiated by the addition of adenosine 3′,5′-monophosphate (2 m*M*) as described (*1*). Reactions were stopped after 5, 10, and 15 minutes by the addition of 0.1 ml of 55% trichloracetic acid and 0.2 ml of acid molybdate solution. After centrifugation (2500 rev/min, 10 minutes), the supernatants were added to deionized water (1 ml) and Fisk-SubbaRow reagent (0.1 ml). Color was allowed to develop for 10 minutes and A_{660} measured for each tube within 10 minutes.

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REFERENCES

1. P. M. Colombani, A. Robb, A. D. Hess, *Science* **228**, 337 (1985).
2. M. M. Merker and R. E. Handschumacher, *J. Immunol.* **132**, 3064 (1984); R. E. Handschumacher *et al.*, *Science* **226**, 544 (1984).
3. P. Hiestand, personal communication.
4. H. Hidaka *et al.*, *Mol. Pharmacol.* 17, 66 (1980).
5. J. D. Johnson and L. A. Wittenauer, *Biochem. J.* **211**, 473 (1983).
6. P. M. Colombani, E. C. Bright, A. D. Hess, *Transplant Proc.*, in press.
7. A. D. Hess *et al.*, *ibid.*, in press.
8. P. M. Colombani and A. D. Hess, in preparation; R. J. Tesi *et al.*, *Surg. For.* **26**, 339 (1985).

3 December 1985; accepted 3 March 1986