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ALLOSTERIC INTERACTIONS AMONG DRUG BINDING SITES ON CALMODULIN

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Felodipine is a fluorescent dihydropyridine Ca^{2+}-antagonist. It binds to calmodulin in a Ca^{2+}-dependent manner, and undergoes a fluorescence increase which allows us to monitor its interaction with calmodulin. Hydrophobic ligands including the calmodulin antagonist, R24571 and Ca^{2+} antagonists, prenylamine and diltiazem, bind to calmodulin and potentiate felodipine binding by as much as 20 fold. These studies suggest that allosteric interactions occur among different drug binding sites on calmodulin. Our results are discussed in terms of the mechanism of action of calmodulin.

The Ca^{2+} dependent regulatory protein calmodulin (CDR) modulates a wide variety of Ca^{2+} dependent events in the cell including smooth muscle contraction, glandular secretion, neurosecretion, and some aspects of cell-cell interactions and cell proliferations. (1-4) Ca^{2+} binding to CDR produces large structural changes including the formation of 3-4 ligand binding sites which are hydrophobic in nature and bind a variety of hydrophobic ligands including CDR-antagonists and some Ca^{2+}-antagonists (5-9). Some of these same sites are thought to be the sites where CDR binds and activates various proteins in a Ca^{2+}-dependent fashion (5,6,8). Calmodulin antagonists can inhibit CDR mediated events by displacing or disrupting the complex formed between CDR and some of the proteins it activates (6,8,9).

Questions have been raised as to how one protein can regulate so many diverse Ca^{2+}-dependent events with any degree of specificity. Haiech

ABBREVIATIONS:
CDR for calmodulin, CDR_{DANS} for dansylchloride labeled calmodulin, R24571 for 1-[bis(p-chlorophenyl)methyl] -3-[2,4-dichloro-β-(2,4 dichlorobenzyloxyl phenethyl] imidazoliumchloride. Felodipine for [4-(2,3-dichlorophenyl)-1,4-dihydropyridine-2,6-dimethyl 3,5-dicarboxylic 3-ethylester and 5-methylester)] .

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et.al. have presented evidence for an ordered binding of Ca^{2+} ions which could activate CDR for specific functions as a function of intracellular $[Ca^{2+}]$ (10). A second possibility is that the Ca^{2+}-dependent hydrophobic binding sites on calmodulin undergo allosteric interactions among themselves. To test this possibility, we have studied the effect of various calmodulin antagonists and Ca^{2+} antagonists on the binding of the dihydropyridine felodipine to calmodulin. Felodipine was chosen because it is the fluorescent member of this powerful class of Ca^{2+}-antagonist-vasodilators and is known to bind 1-2 sites on CDR with 10^{-5}-10^{-6}M affinity (11).

METHODS

Calmodulin was purified from bovine testis, Ca^{2+} titrated in EGTA buffer systems, and labeled with dansylchloride as previously described (7). Felodipine was excited at 365nm and its fluorescence was monitored at 445nm. Drug titrations and fluorescence studies were done by procedures previously (7,8) described and as in the figure legends.

RESULTS AND DISCUSSION

Felodipine is weakly fluorescent in aqueous solution and its fluorescence is increased when it binds to calmodulin. Fig. 1 shows the fluorescence spectra of felodipine (curve 1). Addition of calmodulin and of $MgCl_2$ produce no change in its spectra. The subsequent addition of $CaCl_2$ however, produces a large (>2 fold) fluorescence increase (curve 2). This suggests that Ca^{2+} binding to Ca^{2+} specific sites on calmodulin facilitate felodipine binding. Addition of prenylamine produces a further increase in felodipine fluorescence (curve 3), only in the presence of both Ca^{2+} and CDR. Since the increase in felodipine fluorescence occurs with its binding to CDR, these studies suggest that prenylamine can potentiate felodipine binding to calmodulin.

We have tested several calmodulin antagonists and Ca^{2+} antagonists for their ability to potentiate felodipine binding to calmodulin. Drug titrations of 1μM felodipine, 0.2μM calmodulin are shown in Fig. 2. Each drug produces a fluorescence enhancement at low concentrations followed by

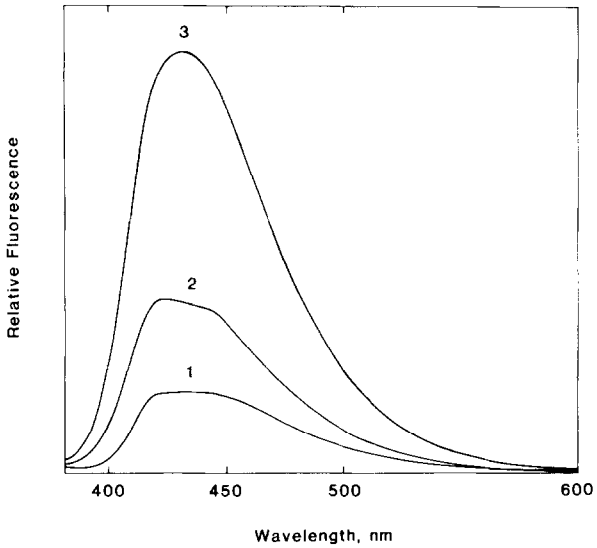

Fig. 1 Effect of Ca^{2+} and prenylamine on calmodulin-felodipine fluorescence. Fluorescence spectra of 1μM felodipine (curve 1) in 10 mM MOPS, 90 mM KCL, 2mM EGTA, pH 7.0 buffer followed by additions of 2μM calmodulin (curve 1), and 3mM $MgCl_2$ (curve 1). The subsequent addition of 3mM $CaCl_2$ (pCa 3.0, pH 7.0) produced the increase shown in curve 2 and the subsequent addition of 50μM prenylamine produced the increase shown in curve 3.

its reversal at higher concentrations. The increase with R24571, prenylamine and diltiazem are half-maximal at $6x10^{-8}$M, 1μM, and 200μM, respectively. Previously, we have reported a dansylated calmodulin, CDR_{DANS}, which undergoes a fluorescence increase with the Ca^{2+}-dependent binding of drugs and proteins (7). Fig. 2 shows, for comparison, drug titrations of 0.2μM CDR_{DANS}. Clearly, over the same concentration range where each drug binds CDR_{DANS} it binds and produces fluorescence increases in calmodulin-felodipine solutions. These fluorescence increases result from drug binding to sites (other than the felodipine binding site) on calmodulin and causing an increase in the number and/or affinity of felodipine binding sites. The fluorescence decreases occur after saturation of these higher affinity drug binding sites (after CDR_{DANS} fluorescence has leveled) and presumably result from drug binding to calmodulin and displacing felodipine from its binding sites. In each case, drug titrations of calmodulin-felodipine in the absence of Ca^{2+} (pCa>10, 2mM EGTA) or of felodipine alone

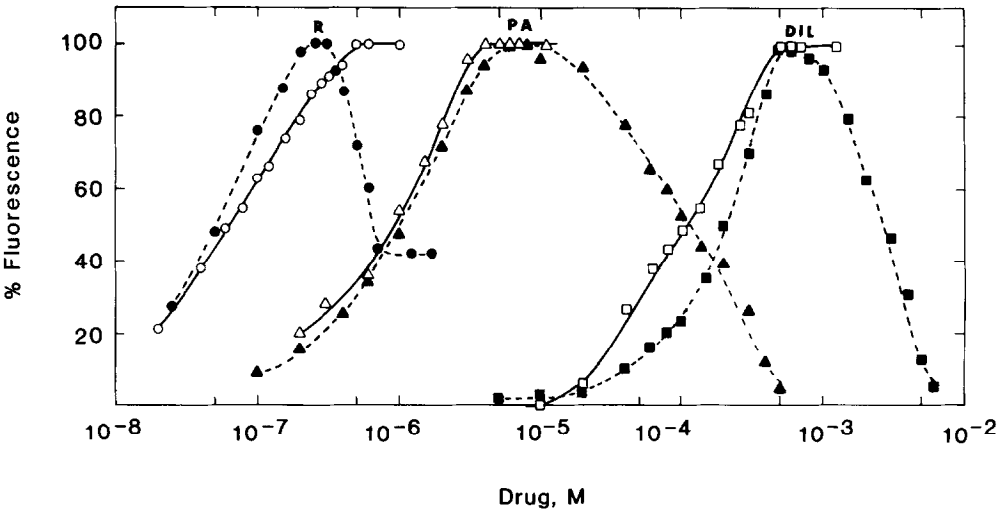

Fig. 2 Effect of Ca^{2+} antagonists and calmodulin antagonists on felodipine-calmodulin and CDR_{DANS} fluorescence. The percent of the total change in the fluorescence of felodipine-calmodulin is shown as a function of added R24571 (●—●—●), prenylamine (▲—▲—▲), and diltiazem (■—■—■). The total fluorescence increase was ~1.4 fold in each case. Each cuvette contained $2x10^{-7}$M calmodulin, $1x10^{-6}$M felodipine in the buffer of Fig. 1 at pCa 3.0 The percent of the total increase in CDR_{DANS} fluorescence as a function of added R24571 (○—○—○), prenylamine (△—△—△) and diltiazem (□—□—□) is shown for comparison. These solutions contained $2x10^{-7}$M CDR_{DANS} in the above buffer.

(with or without Ca^{2+}) produced no fluorescence change. These data are consistent with drug binding to calmodulin at one type of Ca^{2+}-dependent binding sites being responsible for potentiating felodipine binding to calmodulin at other binding sites.

We have titrated felodipine with calmodulin to determine its apparent binding constant in the presence of these potentiating Ca^{2+} antagonists and calmodulin antagonists (Fig. 3). In the absence of added drug, CDR produces a 2.2 fold fluorescence increase which is half-maximal near 8.5μM CDR. This is in good agreement with the Kd of 3 μM that we have determined by CDR_{DANS} fluorescence (7) and with the Kd of 1-10μM determined by NMR (11). Some drugs which bind to calmodulin potentiate this CDR induced fluorescence increase allowing it to occur at lower CDR concentrations. R24571 and prenylamine were most effective of the drugs tested (Fig. 3). The fluorescence increases were half-maximal at 0.4μM, 0.55μM, 1.7μM, 3.6μM for prenylamine, R24571, diltiazem, and verapamil, respectively.

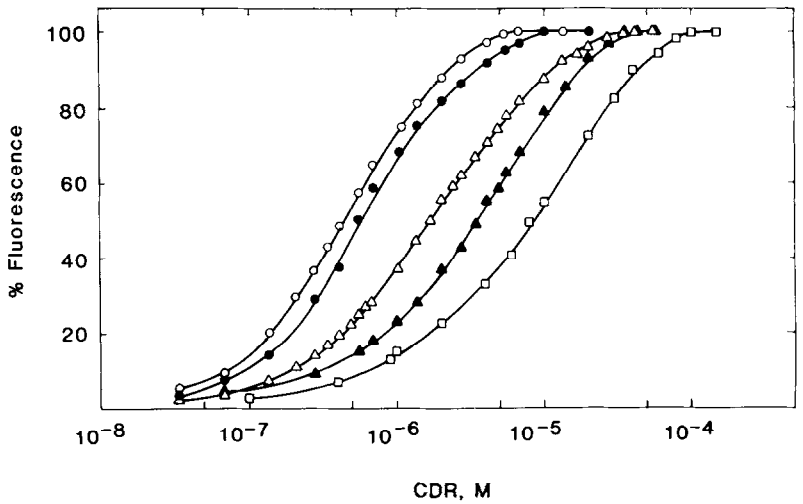

Fig. 3 Effect of Ca^{2+} antagonists and calmodulin antagonists on felodipine binding to calmodulin. The percent of the total fluorescence increase in felodipine as a function of added calmodulin is shown in the presence of 10μM prenylamine (○—○—○), 2μM R24571 (●—●—●), 500μM diltiazem (△—△—△), 200μM verapamil (▲—▲—▲) and in the absence of added drug (□—□—□). The total fluorescence increases were 5.0, 3.1, 10.8, 4.6, and 2.2 fold respectively. Each cuvette contained $5x10^{-7}$M felodipine in the pCa 3.0 buffer described in Fig. 2 legend and was titrated with calmodulin in the presence or absence of drug.

The concentrations of these drugs, used in Fig. 3, were the concentrations which gave the greatest shift in the half-maximal fluorescence increase produced by calmodulin binding and these concentrations agree very well with the concentrations of drug which give the maximal fluorescence increase in Fig. 2 (i.e. The concentration of drug which saturates the higher affinity binding sites on calmodulin.) Lower concentrations of each drug could, however, produce substantial shifts in the apparent CDR-felodipine affinity. For example, although 2μM R24571 was necessary for the maximal potentiation of felodipine binding, $5x10^{-7}$M R24571 could shift the apparent Kd quite substantially (from 8.5μM to 1μM).

In the absence of Ca^{2+} (pCa>10, 2mM EGTA), calmodulin produced no fluorescence increase in felodipine under the conditions of Fig. 3. Titrations of lower felodipine concentrations ($5x10^{-8}$and $1x10^{-7}$M, in the presence of Ca^{2+}) produced no shift in the curves of Fig. 3, suggesting that the $K_{apparents}$ may be taken from the midpoints of the curves in Fig.3

These data indicate that some hydrophobic drugs can bind to calmodulin in a Ca^{2+} dependent manner and increase its apparent affinity for felodipine. Our control studies show that none of the potentiating drugs bind to free felodipine to produce any fluorescence increase. In fact, both Ca^{2+} and calmodulin are required before these potentiating drugs can produce an increase in felodipine fluorescence. Further, each potentiating drug produces the increase in CDR-felodipine fluorescence over the same concentration range that it binds to calmodulin. Thus, drug binding to certain sites on calmodulin can enhance felodipine binding (either number and/or affinity of felodipine binding sites) to other sites on calmodulin through an allosteric mechanism. This represents the first evidence that allosteric interactions occur among the various hydrophobic ligand binding sites on calmodulin. Since these same sites are thought to be the sites where CDR binds and activates various proteins, it is likely that allosteric interactions occur among the various protein binding sites of calmodulin. If this is the case, then calmodulin's activation of a specific protein could be regulated not only by Ca^{2+}, but also by other proteins which bind calmodulin and are perhaps themselves activated by calmodulin. Thus, the ability of CDR to activate certain proteins may depend on the degree of occupancy of its Ca^{2+} binding sites and its hydrophobic ligand binding sites. It is also possible that endogenous hydrophobic ligands (as well as calmodulin binding proteins) could serve to regulate (either activate or deactivate) Ca^{2+} loaded CDR within the cell through such allosteric mechanisms. These possibilities remain to be tested.

The hypothesis of allosteric interactions among the hydrophobic binding sites of calmodulin is certainly not exclusive of the ordered Ca^{2+} binding hypothesis of Haiech et al. (10). Control of action of calmodulin, both by Ca^{2+} and by the occupancy of its various hydrophobic binding sites, may indeed be necessary to explain how this protein regulates so many diverse Ca^{2+} dependent phenomena with such apparent harmony. Further, allosteric interactions among drug binding sites on other Ca^{2+} binding

proteins, including the voltage dependent Ca^{2+} channel, have recently been reported (12). These allosteric interactions may,therefore, represent a common mechanism for control of the actions of Ca^{2+} binding proteins.

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