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Cooperativity among Calmodulin's Drug Binding Sites†

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ABSTRACT: The binding of felodipine, a dihydropyridine Ca^{2+} antagonist, to calmodulin has been studied by equilibrium dialysis and fluorescence techniques. Analysis using the Hill equation gives a Hill coefficient of 2. A plot of bound [felodipine] vs. free [felodipine]^2 gives a B_{max} of 1.9 mol/mol and a $K_{0.5}$ of 22 μM. Two calmodulin antagonists, prenylamine and R24571, which have previously been shown to potentiate the fluorescent enhancement observed when felodipine binds to calmodulin [Johnson, J. D. (1983) *Biochem. Biophys. Res. Commun. 112*, 787], produce a reduction in Hill coefficient to 0.7 and 1.0, respectively, and account for the observed potentiation of felodipine binding. Titrations of felodipine with calmodulin in the absence and presence of prenylamine and R24571 suggest that these drugs decrease the $K_{0.5}$ of calmodulin for felodipine by 25-fold. Thus, potentiating drugs (prenylamine and R24571) bind to either of the two felodipine binding sites and, through an allosteric mechanism, result in felodipine binding to the remaining site with greatly enhanced affinity. Two types of potentiating drugs are observed. Prenylamine exhibits a Hill coefficient of 0.8 whereas felodipine, R24571, and diltiazem exhibit Hill coefficients of 2 in their potentiation of felodipine binding. Titrations of felodipine and calmodulin with Ca^{2+} exhibit cooperativity with a Hill coefficient of 4. Half-maximal binding occurs near pCa 6.0. In the presence of R24571, the calcium dependence of felodipine binding is biphasic, now exhibiting a much higher affinity (pCa 7.6) component. A model is presented to explain the relationship of these various allosterically regulated conformers of calmodulin and their interactions and activation with its target proteins.

Calmodulin is a ubiquitous calcium binding protein that binds 4 mol of calcium/mol of protein and undergoes large calcium-dependent changes in structure. These structural changes form or expose hydrophobic binding sites on its surface (Laporte et al., 1980; Tanaka & Hidaka, 1980) where calmodulin binds and activates as many as 30 different proteins in a calcium-dependent manner [see Klee et al. (1980) for review]. Certain drugs, including trifluoperazine (TFP), W-7, and R24571, can bind to these sites with high to moderate affinity and inhibit calmodulin's interaction and activation of many of the proteins that it modulates. Presently, it is uncertain how calmodulin can bind and selectively activate so many different proteins with any degree of specificity.

Several models have been proposed to explain how calmodulin might exhibit selectivity as a calcium-dependent modulator. For example, some target proteins appear to exhibit

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different $K_{0.5}$ values for calmodulin (Scharff, 1981). Further, the calmodulin species that activates the target may vary as a function of [Ca^{2+}]. Haiech et al. (1981) have provided evidence for an ordered binding of calcium that might activate CaM for specific functions dependent upon the free Ca^{2+} ion concentration. In addition, different proteins may interact with different binding sites on calmodulin, allowing some degree of selective activation. All of the above mechanisms may contribute to the selectivity of CaM in activating its various target proteins.

Previously, we have shown that the dihydropyridine calcium antagonist felodipine binds to calmodulin in a calcium-dependent manner and that this binding (fluorescence) can be enhanced by the interaction of calmodulin antagonists at distinct sites via allosteric mechanisms (Johnson, 1983). We have suggested that these allosteric interactions among the drug/protein sites on calmodulin might provide a mechanism for selectively directing calmodulin to specific target proteins.

In this model, endogenous regulators (chemicals, peptides, or proteins) would bind to calmodulin and alter its conformation via these allosteric mechanisms. These various calmodulin conformers would have an altered protein binding site that would now exhibit enhanced selectivity for activating certain target proteins over others.

In an effort to more fully understand the mechanism of the allosteric interactions among the drug binding sites on calmodulin, we have analyzed the binding to felodipine to calmodulin in the presence and in the absence of its potentiating drugs. We propose a model to explain how cooperativity among calcium-dependent drug/protein binding sites may work in concert with the cooperativity among the calcium binding sites to produce distinct conformers of calmodulin for selective activation of its target proteins.

Experimental Procedures

Equilibrium Dialysis. Buffer used was 10 mM Mops,[1] pH 7.0, 2 mM EGTA, 3 mM $CaCl_2$, and 90 mM KCl (buffer A). [^{3}H]Felodipine of constant specific activity was added to the solution (50 mL) outside the dialysis bag, which contained 3 mL of 1 μM calmodulin. Specific activity was determined by using a millimolar extinction coefficient at 370 nm of 6.4 in 95% ethanol.

Fluorescence Enhancements. Fluorescence measurements were carried out in buffer A with 0.2 μM calmodulin and 1 μM felodipine and varying amounts of calmodulin antagonists. For titrations with felodipine (dissolved in ethanol), buffer A was used with ethanol held fixed at 4%. For felodipine titrations in the presence of prenylamine or R24571, the order of addition was felodipine, calmodulin, and then R24571 or prenylamine. Fluorescence measurements were carried out as previously described (Johnson, 1983), free [Ca] was determined in EGTA–Ca^{2+} buffer systems as previously described (Johnson et al., 1978), and calmodulin concentrations were determined by the biuret method. Hill plots were analyzed by an iterative linear regression Hill plot program, and B_{max} was determined by the best fit of the data to the Hill equation.

Felodipine and [^{3}H]felodipine were gifts of A. B. Hässle Pharmaceutical, Mölndal, Sweden. R24571 was obtained from Janssen Laboratories. Prenylamine and diltiazem were obtained from Chinion and Marion Pharmaceuticals, respectively. All other chemicals were reagent grade. Calmodulin was purified from bovine testes as previously described (Crouch et al., 1981).

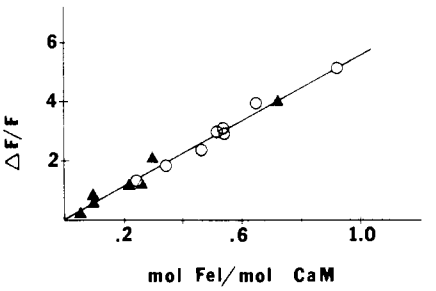


FIGURE 1: Correlation of the fluorescence increase observed in felodipine with its binding to CaM. The change in felodipine fluorescence is plotted vs. [^{3}H]felodipine binding in the presence (○) and absence (▲) of 10 μM prenylamine. Equilibrium dialysis was conducted in buffer A with 1 μM calmodulin and variable (0.5–5 μM) felodipine.

Results

To determine whether the previously reported increase in felodipine fluorescence observed upon the addition of prenylamine to calmodulin–felodipine was due to enhanced felodipine binding or increased quantum yield of bound felodipine, equilibrium dialysis was carried out at varying concentrations of felodipine in the absence or presence of 10 μM prenylamine (PA). This concentration of PA has previously been shown to produce optimal enhancement (Johnson, 1983). A plot of [^{3}H]felodipine binding vs. the fluorescence difference between inside and outside of the dialysis bag (Figure 1) shows that the quantum yield is enhanced 5.6-fold when 1 mol of felodipine is bound per mole of calmodulin, both in the presence and in the absence of prenylamine. The increase in fluorescence produced by prenylamine is, therefore, due to enhanced felodipine binding. Further, this curve shows the close correlation between felodipine binding and its fluorescence enhancement in both the presence and the absence of potentiating drug and was subsequently used to quantitate felodipine binding.

A Scatchard plot of the fluorescent data obtained from felodipine titrations of calmodulin (B vs. B/S^2) gave a straight line (indicating that the Hill coefficient was 2) with a B_{max} of 1.9 mol/mol and a $K_{0.5}$ of 22 ± 4 μM (r = 0.90) (Figure 2A). Scatchard analysis in the presence of 10 μM prenylamine showed a reduction of both $K_{0.5}$ ($K_{0.5}$ = 4 μM) and B_{max} (B_{max} = 0.8), whereas neither $K_{0.5}$ or B_{max} was altered appreciably by 2 μM R24571. Figure 3 shows Hill plots (B_{max} = 1.9) of calmodulin titrated with felodipine alone and in the presence of 2 μM R24571 or 10 μM prenylamine, concentrations that optimally enhance felodipine–calmodulin fluorescence. These Hill plots show that the fluorescence enhancement results from a reduction in the Hill coefficient from 2 to 1 for R24571 and from 2 to 0.6 for prenylamine.

In an effort to determine the effects of prenylamine and R24571 on the $K_{0.5}$ of calmodulin for felodipine, we have conducted titrations of felodipine (±prenylamine or R24571) with calmodulin. Hill plots of these titrations are shown in Figure 4. Hill coefficients of 1.1 and 1.0 were obtained in the presence of R24571 and prenylamine, respectively, compared to 0.8 with felodipine alone. The data indicate that both prenylamine and R24571 decrease the apparent $K_{0.5}$ of calmodulin for felodipine from 14 μM to 0.5 μM and 0.6 μM, respectively. Thus, the data from Figures 3 and 4 indicate that prenylamine and R24571 reduce the Hill coefficient from 2 to 1 or less and enhance the affinity of calmodulin for fel-

[1] Abbreviations: ANS, anilinonaphthalenesulfonate; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

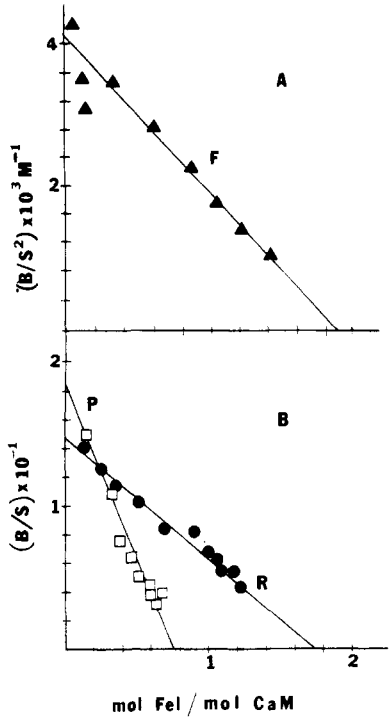


FIGURE 2: (A) Scatchard analysis (B/S^2 vs. B) from fluorescence data of felodipine titrations of 1 μM calmodulin. Bound felodipine was determined from a standard curve of felodipine fluorescence vs. [^{3}H]felodipine bound as in Figure 1. Buffer was 4% v/v ethanol in buffer A. (B) Scatchard analysis (B/S vs. B) in the presence of 10 μM prenylamine (□) or 2 μM R24571 (●). Excitation was at 365 nm, and felodipine emission was measured at 445 nm. Titrations were done in buffer A with 4% v/v ethanol. Nearly identical data were obtained when these titrations were conducted with minimal ethanol (0.2%) present except that the $K_{0.5}$ was decreased 2-fold in both the absence and presence of potentiating drug.

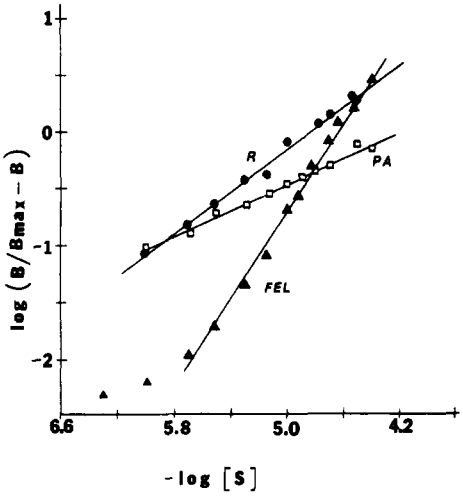


FIGURE 3: Hill plot analysis of fluorescence enhancement observed when 1 μM calmodulin is titrated with felodipine alone (▲) ($K_{0.5}$ = 22 μM, slope = 1.9, r = 0.997), in the presence of 2 μM R24571 (●) ($K_{0.5}$ = 15 μM, slope = 1.0, r = 0.993), or in the presence of 10 μM prenylamine (□) ($K_{0.5}$ = 58 μM, slope = 0.6, r = 0.993). For felodipine alone, only those points where $S > 5$ μM were used in determination of B_{max} and the Hill coefficient. Conditions and data were the same as in Figure 2.

odipine at the allosterically affected felodipine site by approximately 25-fold.

The fluorescence increases and decreases observed when felodipine–calmodulin is titrated with prenylamine, R24571, and diltiazem are compared with a theoretical curve in Figure 5 (upper panel). The F curve (▲) shows the fluorescent enhancement plot one would expect to see if one titrated cal-

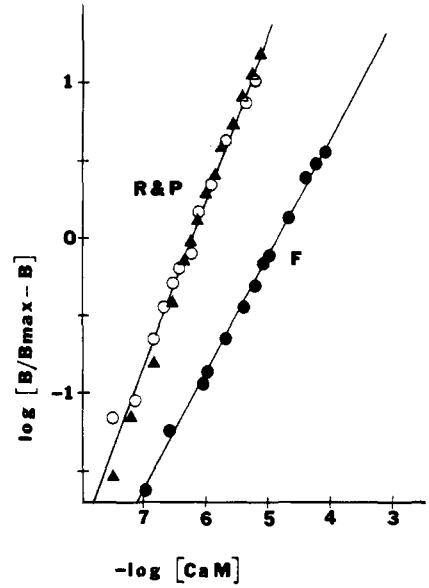


FIGURE 4: Hill plot analysis of fluorescence enhancement of felodipine titrated with calmodulin alone (●) or in the presence of 2 μM R24571 (▲) or 10 μM prenylamine (○). The cuvette contained 0.5 μM felodipine in buffer A, and [CaM] represents total calmodulin added.

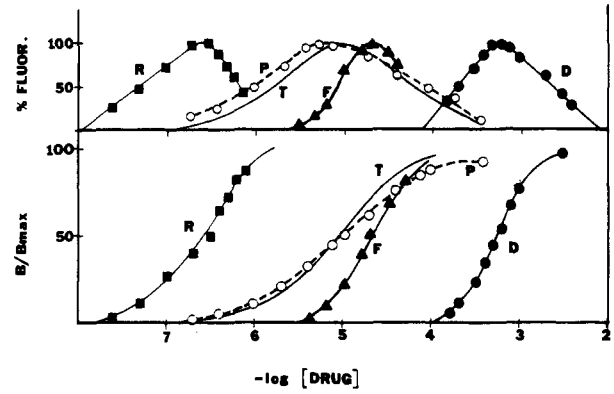


FIGURE 5: (Upper panel) The fluorescence changes produced by titrations of calmodulin–felodipine with R24571 (■), prenylamine (○), and diltiazem (●) are compared with a theoretical curve. The solid line (T) is the curve expected if felodipine binds to calmodulin to which one and only one molecule of potentiating drug has bound to one of two equivalent sites on calmodulin (K_d = 10.7 μM). The F curve (▲), a plot of percent bound vs. bound at 1 μM felodipine, represents a titration of 1 μM felodipine and 1 μM calmodulin with a drug that is identical to felodipine but is nonfluorescent. The drug titrations were conducted with 0.2 μM calmodulin and 1 μM felodipine in buffer A. (Lower panel) Plots of the integrals of the theoretical (—), felodipine (▲), R24571 (■), prenylamine (○), and diltiazem (●) curves shown in the upper panel. Hill plots of the integral of the theoretical curve gave a slope of 1.0, r = 0.9999, with $K_{0.5}$ = 9.7 μM. Hill coefficients were 1.8 (r = 0.990) for R24571, 2.0 (r = 0.999) for diltiazem, and 0.8 (r = 0.999) for prenylamine titrations of calmodulin–felodipine. The slope and $K_{0.5}$ determined for the curve of felodipine alone were nearly identical with those experimentally obtained in Figure 3 (K_d = 22 μM, slope = 2.0), verifying integration by the trapezoidal method.

modulin–felodipine (1 μM) with a nonfluorescent analogue of felodipine that had the same $K_{0.5}$ as felodipine. This is represented by a plot of percent felodipine bound minus percent felodipine bound at 1 μM (data taken from Figure 2A and 100% fluorescence = 4% felodipine bound). Also shown is the theoretical (T) fluorescence enhancement one would expect from a titration of calmodulin–felodipine with a drug that has two equivalent sites on calmodulin, assuming that felodipine binds only to those calmodulin molecules that have one and only one drug molecule bound. The plots of $\int_{-\infty}^{\log S}\Delta F\ \mathrm{d}(\log S)/\int_{-\infty}^{+\infty}\Delta F\ \mathrm{d}(\log S)$ vs. log S, shown in Figure 5 (lower panel),

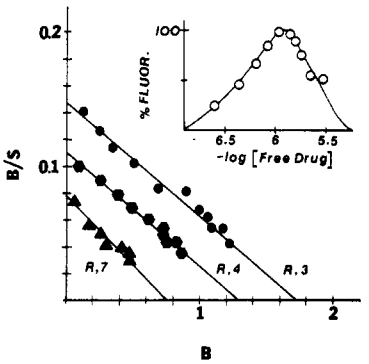


FIGURE 6: Scatchard analysis of felodipine binding to calmodulin in the presence of R24571 at various calcium concentrations. Concentrations of 1 μM calmodulin and 2 μM R24571 were titrated with felodipine in 10 mM MOPS, 90 mM KCl, 2 mM EGTA, and 4% ethanol with free calcium adjusted to pCa 7.0 (▲), 4.0 (●), or 3.0 (●). $K_{0.5}$ values were 10 μM (r = 0.94), 12 μM (r = 0.98) and 12 μM (r = 0.97), respectively. The inset shows the fluorescence increase produced when 0.5 μM calmodulin and 1 μM felodipine at pCa 7.0 are titrated with R24571. A Hill plot of the integral showed $K_{0.5}$ = 1 μM: Hill coefficient = 2.0; r = 0.998.

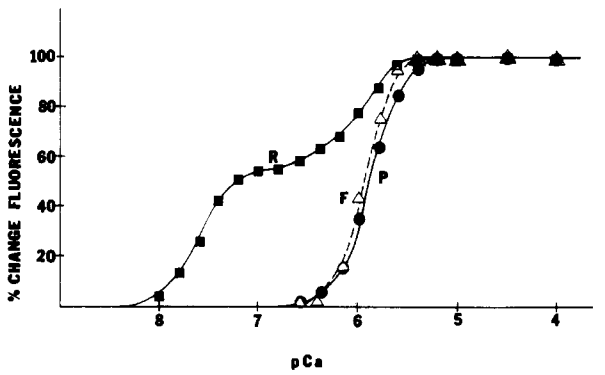


FIGURE 7: Calcium titrations of 2 μM calmodulin and 1 μM felodipine (△), calmodulin, felodipine, and 10 μM prenylamine (●), or calmodulin, felodipine, and 2 μM R24571 (■). Hill coefficient with felodipine alone was 3.8 ■ 0.3; r = 0.993 (average of five determinations). Hill coefficient for felodipine and prenylamine = 2.5 ± 0.1; r = 0.998 (average of three determinations). Buffer contained 10 mM MOPS, 90 mM KCl, and 2 mM EGTA, pH 7.0, and $CaCl_2$ was added to give the free Ca^{2+} indicated.

give the expected S-shaped drug titration plots. Prenylamine is most similar to the theoretical two equivalent site model (Hill coefficient = 0.8), indicating that felodipine binds with high affinity to those calmodulin molecules containing one and only one prenylamine but that prenylamine does not potentiate its own binding. Both diltiazem and R24571 are similar to what one would expect for nonfluorescent analogues of felodipine and exhibit Hill coefficients near 2.0. These latter drugs potentiate binding of both felodipine and themselves to a second allosterically regulated site.

Therefore, there appear to be at least two classes of drugs that bind to calmodulin. Class 1: Those drugs that potentiate felodipine binding to a second site but have similar affinities for both sites themselves, exhibiting Hill coefficients of 1 or less. Class 2: Drugs that exhibit positive cooperativity (Hill coefficient = 2) and potentiate binding of both themselves and felodipine to a second site.

Our studies illustrate another major difference between PA and R24571. Although PA, R24571, and diltiazem can each potentiate felodipine binding at pCa 3.0, at much lower [Ca^{2+}] only R24571 is an effective potentiator. Figure 6 (inset) shows the effect of R24571 on calmodulin–felodipine fluorescence at pCa 7.0. At pCa 7.0, the $K_{0.5}$ of R24571 for CaM–felodipine is estimated to be 1 μM, and it again enhances CaM–felodipine fluorescence with a Hill coefficient of 2.0 (r = 0.998). At pCa 7.0 neither prenylamine (at 100 μM), diltiazem (at 500 μM), nor felodipine itself (at 10 μM) will potentiate felodipine binding to calmodulin. Figure 6 shows Scatchard analysis of felodipine titrations of calmodulin in the presence of R24571 at pCa 7.0, 4.0, and 3.0. These data show a reduction in B_{max} at lower Ca^{2+} concentrations. This is consistent with a reduction in the number of active CaM molecules at lower [Ca^{2+}]. No fluorescence enhancement was noted at pCa 7 with felodipine alone or in the presence of 10 μM prenylamine. Figure 7 shows the calcium dependence of the fluorescence increase produced by felodipine binding to calmodulin. This increase is half-maximal at pCa 6.0 with a Hill coefficient of 4. The addition of 2 μM R24571 dramatically influences the calcium dependence of these fluorescence changes. In the presence of R24571, the calcium curve is clearly biphasic, and felodipine binding is potentiated by calcium binding to sites with $K_{0.5}$ estimated to be pCa 7.6 and pCa 6.0. The presence of 10 μM PA has little effect on calcium dependence of felodipine–calmodulin, but the Hill coefficient was reduced from 4 to 3. These data suggest that at pCa 7.0, R24571 but not 10 μM prenylamine can potentiate felodipine binding to calmodulin and that, in the presence of felodipine, R24571 but not prenylamine can enhance the calcium affinity of some of the calcium binding sites on CaM.

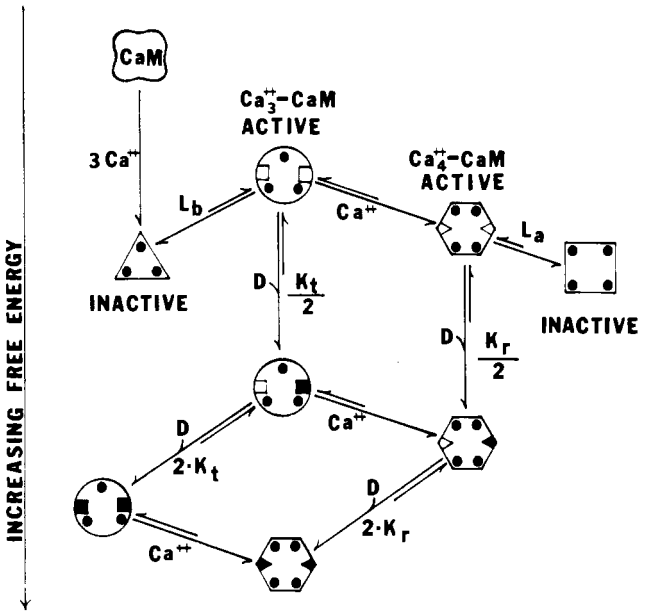
Discussion

Previously, we have shown that calcium binding to CaM exposes hydrophobic binding site(s) for felodipine (Johnson, 1983). Felodipine binding can be followed by the large increase in its fluorescence, which occurs concomitant with its binding to CaM. Further, we have presented the first evidence that some of the drug binding sites on CaM are allosterically related (Johnson, 1983). Some calmodulin antagonists, R24571 and PA, could bind to CaM at low concentrations and enhance felodipine binding and fluorescence while at high concentrations they could apparently displace felodipine and decrease its fluorescence. In this study we have explored the mechanism for allosteric drug effects on calmodulin–felodipine fluorescence in a quantitative manner.

We find that CaM has two binding sites for felodipine ($K_{0.5}$ = 22 μM) and that these sites exhibit strong positive cooperativity (Hill coefficient = 2.0). Calmodulin antagonists (CaM ANTs), such as prenylamine and R24571, enhance felodipine binding by decreasing the Hill coefficient for its binding from 2.0 to 0.6 and 1.0, respectively. These nonfluorescent CaM ANTs presumably bind to one of the two felodipine binding sites and potentiate felodipine binding to the remaining felodipine binding site. Our titrations of felodipine with calmodulin in the presence of its potentiators (prenylamine and R24571) show that felodipine now binds to a single site whose dissociation constant has been reduced from 14 μM to 0.5 μM and 0.6 μM, respectively. Thus, prenylamine and R24571 enhance felodipine–calmodulin fluorescence by binding to a single site on calmodulin and allosterically enhancing the affinity of felodipine at a second binding site.

Felodipine binding and its potentiation by prenylamine and R24571 are strictly dependent on calcium. Under low Ca^{2+} conditions (pCa 10–7.0), felodipine will not bind, and prenylamine (100 μM) will not potentiate its binding as it does at pCa 3.0 or 4. R24571, however, can potentiate felodipine binding even at pCa 7.0. R24571 in the presence of felodipine produces an apparent increase (40-fold) in the affinity of some of the calcium binding sites that regulate felodipine binding (Figure 7). This suggests that prenylamine and R24571 ex-

Scheme I: Model for Calmodulin Interactions with Calcium and Drugs[a]



[a] L_a and L_b represent the ratio of inactive species to active species for Ca_4CaM and Ca_3CaM, respectively. K_r and K_t are dissociation constants. D, drug; (•) binding site occupied with calcium; (□) and (◇), unoccupied exposed drug binding sites of distinct conformations; (■) and (◆), occupied binding sites of these distinct conformations.

hibit some fundamental differences in their binding and that only R24571 can enhance calcium binding to CaM such that felodipine will bind at pCa 7.0. The potentiation of felodipine binding at pCa 7.0 by R24571 may be due to its high affinity for calmodulin or may reflect some selectivity in its binding. Johnson & Wittenauer (1983) have reported at $K_{0.5}$ of 2–3 nM for R24571 and dansylated calmodulin. Therefore, of the drugs tested, only R24571 can bind to calmodulin with high enough affinity to pull the reaction toward the CaM–Ca^{2+}–R24571 state at low Ca^{2+}. R24571 is apparently unique among the drugs tested in that at low concentrations it can increase the affinity of CaM for calcium so that Ca^{2+}-dependent felodipine binding can occur even at pCa 7.0.

In the following model (see Scheme I) we account for the Ca^{2+}-dependent allosteric interactions among prenylamine, R24571, and felodipine binding sites. Kuo (1983) has shown that, in the Monod–Wyman–Changeaux (Monod et al., 1965) model, cooperative ligands could be both activators and inhibitors. The sigmoidal curve (Hill coefficient = 2.0) of a cooperative ligand shifts to a hyperbolic curve (Hill coefficient = 1.0) with enhanced binding at low ligand concentration in the presence of a second cooperative ligand. This is analogous to what we observe with R24571 and prenylamine, respectively, in their enhancement of felodipine binding. Noncooperative ligands cannot, by this model, produce enhancement. Therefore, enhancement of felodipine binding implies cooperative binding. The apparent negative cooperativity of prenylamine cannot be explained by a simple two-state Ca^{2+}_4–CaM model. This apparent negative cooperativity can best be explained by prenylamine's ability to bind to an active Ca^{2+}_3–CaM conformer, which is in equilibrium with an active Ca^{2+}_4–CaM conformer (Scheme I). This would keep the concentration of the Ca^{2+}_4–CaM conformer relatively constant and produce apparent negative cooperativity (Hill coefficient = 0.6) when CaM was titrated with felodipine in the presence of prenylamine. This two active conformer model could also explain the broad curve observed when CaM–felodipine is titrated with prenylamine (Hill coefficient = 0.8) by the same mechanism.

A free-energy coupling model in which both Ca^{2+}_3–CaM and Ca^{2+}_4–CaM have active conformations is the simplest model to explain the observed results (see Scheme I). In this model prenylamine could bind the Ca^{2+}_3–CaM conformer almost as well as to Ca^{2+}_4–CaM conformer whereas diltiazem, R24571, and felodipine would have a greater preference for the Ca^{2+}_4–CaM conformer. When calmodulin and felodipine are titrated with Ca^{2+} a Hill coefficient of 4 is observed, consistent with Ca^{2+}_4–CaM being the active species. Ca^{2+} titrations of CaM in the presence of both felodipine and prenylamine exhibit Hill coefficients of 3 or less, consistent with prenylamine binding to Ca^{2+}_3–CaM (i.e., for prenylamine, $K_r \sim K_t$; for R24571, diltiazem, and felodipine, $K_r \ll K_t$).

The equation for drug binding when $K_r \ll K_t$ and $S/K_r \gg 1$ is

$$\frac{B}{B_{max}} = \frac{S^2}{S^2 + L_a K_r^2}$$

This is analogous to the Hill equation, and $K_{0.5}$ from the Hill plot = $L_a^{1/2} K_r$.

The maximum value for K_r is similar to the 0.5 μM value determined from a titration of felodipine with calmodulin in the presence of R24571 or prenylamine (Figure 3). The minimum value for L_a then is 1700 (ΔG = 5 kcal/mol). The Hill plot is a reasonable approximation for $S/K_r > 10$ or for $S > 5$ μM.

At low Ca^{2+} concentration, the Ca^{2+}_3–CaM–R24571 species may predominate over Ca^{2+}_4–CaM–R24571 producing apparent noncompetitive inhibition at low calcium concentrations (B_{max} is reduced from 1.8 to 0.8 mol/mol); an analogous situation is seen with prenylamine at pCa 3 (Figure 2B). This could account for the biphasic Ca^{2+} titration curve seen in the presence of R24571 (Figure 7). The first phase of the fluorescence increase probably represents Ca^{2+}-induced formation of the conformer Ca^{2+}_3–CaM with two drugs (D_2). The final increase probably represents the addition of calcium to this species to form Ca^{2+}_4–CaM with two drugs. The biphasic increases in fluorescence intensity observed in this Ca^{2+} titration would represent sequential increases in drug affinity or free energy of the system as shown in Scheme I.

This model is consistent with the observation of Keller et al. (1982), who observed that TFP enhanced the binding of the first three Ca^{2+} but inhibited binding of the fourth, possibly indicative of preferential binding to Ca^{2+}_3–CaM. ANS, on the other hand, increased the cooperativity of Ca^{2+} binding to all four Ca^{2+} sites.

The model we propose for Ca^{2+}-dependent binding of drugs is similar to those proposed for calmodulin binding to its target proteins. In addition, our model accounts for the cooperative binding among drug/protein binding sites exposed on different Ca^{2+} conformers. In a review of the calcium dependence of calmodulin's activation of various proteins, Cox (1984) has shown that Ca^{2+}_3–CaM and/or Ca^{2+}_4–CaM are the active species. Ca^{2+}_4–CaM is apparently the active species for activating the calmodulin-dependent protein kinase of cardiac sarcoplasmic reticulum (Pifl et al., 1984), and its activation by Ca^{2+} displays a Hill coefficient of 4. For phosphorylase kinase, however, Ca^{2+}_3–CaM is the active species (Burger et al., 1983), with both the tightly bound and loosely bound calmodulin molecules binding three Ca^{2+} in the active state, with each exhibiting distinct Ca^{2+} titration curves. This is consistent with our model's two conformational states for Ca^{2+}_3–CaM, each of which should exhibit distinct Ca^{2+} ti-

tration curves. These studies of CaM–protein interaction are analogous to our finding that $Ca^{2+}{}_3$–CaM and $Ca^{2+}{}_4$–CaM are the active species for drug binding. If this is true, then CaM–ANT drugs might be expected to mimic the effects we observe on felodipine binding. Metzger et al. (1982) have shown that prenylamine is able to enhance and then inhibit the velocity of contraction of skinned smooth muscle. Maximal potentiation is observed at 10 μM prenylamine, while higher prenylamine concentrations produce inhibition. The contraction of chemically skinned smooth muscle is dependent on the calmodulin-dependent interaction and activation of myosin light chain kinase (MLCK). This suggests that prenylamine is able to regulate the binding of MLCK to calmodulin in a manner analogous to its allosteric regulation of felodipine binding.

Further, our studies indicate that some CaM ANT's (R24571) can dramatically enhance the apparent affinity of CaM for Ca^{2+}. Similarly, it has been shown that troponin I (Keller et al., 1982), MLCK (Olwin et al., 1984), and melittin (Maulett & Cox, 1983) binding to calmodulin enhances its Ca^{2+} affinity. In this respect also, drug binding seems analogous to protein binding. Our allosteric model suggests that various drugs may inhibit (or stimulate) calmodulin–protein interactions differentially. Consistent with this, Hidaka et al. (1980) have shown that prenylamine inhibits in the order PDE > $Ca^{2+}Mg^{2+}$ATPase > MLCK whereas W-7 inhibits in the order $Ca^{2+}Mg^{2+}$ATPase > MLCK > PDE, indicating that various drugs can show some selectivity in inhibiting various targets. Moreover, his data also showed very little correlation between inhibition of [^{3}H]W-7 binding and enzyme inhibition, indicating that simple competition cannot account for the observed inhibition of enzymes. This and our data clearly suggest that stricter analysis of drug inhibition data is necessary to determine if some of the CaM-ANTs drugs may allosterically or competitively affect various CaM–protein interactions.

Our finding of two binding sites for felodipine is in agreement with Jarret (1984), who labeled calmodulin with the affinity reagent 10-(3-aminopropyl)-2-(trifluoromethyl)-phenothiazine and found that 2 mol of reagent was covalently coupled per mole of calmodulin. Newton et al. (1983) showed that only 1 mol of 2-chloro-10-(3-isothiocyanatopropyl)-phenothiazine was incorporated into calmodulin but that the modified calmodulin was able to bind to cyclic nucleotide phosphodiesterase and inhibit its activation by unmodified calmodulin. They also observed increased rates of incorporation of affinity label when TFP was added at low concentrations, consistent with our observation of allosteric interaction among calmodulin's drug binding sites. Affinity labels should be useful in determining the effects of drug binding on Ca^{2+} affinity without the interference of multiple equilibria, as well as for determination of K_t, the dissociation constant of the activated state for various drugs.

Finally, the model proposed here has much in common with what is known for hemoglobins (Perutz, 1978). The effect of prenylamine and R24571 on felodipine binding is similar to known effects of CO on hemoglobin where CO enhances hemoglobin affinity for O_2 at low oxygen concentrations and displaces it at high oxygen concentrations (Rodkey et al., 1974). If drug (or protein) binding can liberate a proton from calmodulin similarly to that which occurs when O_2 binds to hemoglobin, this might explain how Ca^{2+} affinity is increased when drugs or proteins bind to calmodulin. Also, there might be naturally occurring allosteric regulators of calmodulin, analogous to those known for hemoglobin, which may alter its sensitivity to Ca^{2+} and/or impart selectivity for calmodulin binding to its target proteins.

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