Localization of a Felodipine (Dihydropyridine) Binding Site on Calmodulin†

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ABSTRACT: The fluorescent dihydropyridine calcium antagonist drug felodipine binds to calmodulin (CaM) in a Ca^{2+}-dependent manner. Its binding can be regulated by the interaction of CaM antagonist drugs through allosteric mechanisms [Mills, J. S., & Johnson, J. D. (1985) *Biochemistry 24*, 4897]. Here, we have examined the binding of a nonspecific hydrophobic fluorescent probe molecule TNS (toluidinylnaphthalenesulfonate) and of felodipine to CAM and several of its proteolytic fragments. While TNS interacts with sites on both the amino-terminal half of the protein [proteolytic fragment TR_1C (1–77)] and the carboxy-terminal half [proteolytic fragment TR_2C (78–148)], felodipine binding shows more selectivity. It binds in a Ca^{2+}-dependent manner to the proteolytic fragments TM_1 (1–106) and TR_2E (1–90) but exhibits only weak affinity for TR_1C (1–77) and TR_2C (78–148). Furthermore, felodipine exhibits selectivity over TNS and trifluoperazine (TFP) in blocking the tryptic cleavage between residues 77 and 78. These studies indicate a selective binding of felodipine to a hydrophobic site existing in residues 1–90 and suggest that productive binding requires amino acids in the region 78–90. Although the felodipine binding site is preserved in fragment 1–106, the allosteric interactions between the prenylamine and the felodipine binding sites that are observed with intact CaM are not observed in this fragment. Rather, prenylamine simply displaces felodipine from its binding site on this fragment. Our results are consistent with calmodulin containing not less than two allosterically related hydrophobic drug binding sites. One of these sites (felodipine) appears to be localized in region 1–90 and the other one in region 78–148.

The regulatory protein calmodulin (CaM)[1] has recently become recognized as a "universal" Ca^{2+} binding protein. It is ubiquitously distributed in nature, its primary structure is highly conserved in all tissues and species, and it has been shown to activate more than 30 different proteins in a Ca^{2+}-dependent manner [see Forsén et al. (1986), Klee & Newton, (1985), Manalan & Klee (1984), and Means et al. (1982) for reviews].

CaM is known to have hydrophobic binding sites on its surface that are exposed as Ca^{2+} binds. These sites are thought to be the regions where CaM interacts with the proteins it activates and the sites where CaM antagonists bind to inhibit various CaM-mediated events.

Presently, it is uncertain how CaM can regulate so many different proteins with any degree of specificity. Recently, the three-dimensional crystal structure of calmodulin has been determined (Babu et al., 1985). This work revealed that calmodulin is a dumbbell-shaped molecule composed of two lobes connected by eight turns of hydrophilic α-helix. Each of these lobes contains two calcium binding domains or E–F hands. Tryptic cleavage in the presence of calcium occurs primarily between residues 77 and 78, located in this long central helix of calmodulin, and results in both an amino-terminal (1–77) and a carboxy-terminal (78–148) half of the molecule. These two fragments have still retained many of the properties of the intact protein. For example, they still bind in a Ca^{2+}-dependent fashion to phenyl-Sepharose and to a phenothiazine affinity column (Vogel et al., 1983; Brzeska et al., 1983; Newton et al., 1984) as well as to other hydrophobic probe molecules (Krebs et al., 1984). Moreover, a number of NMR studies have demonstrated that the metal ion binding sites (Andersson et al., 1983a) and the protein folding (Thulin et al., 1984; Aulabaugh et al., 1984; Ikura et al., 1984; Dalgarno et al., 1984), as well as the way in which the phenothiazine compound TFP interacts with its two binding sites on CaM (Thulin et al., 1984), are similar in the two fragments compared to the intact protein. Despite the existence of such structural similarities, addition of the Ca^{2+}-saturated fragments to target proteins, such as the cyclic nucleotide phosphodiesterase, does not result in activation [see Newton et al. (1984) and references cited therein]. This suggests that coordinated interactions between both halves of CaM must occur in order to facilitate activation. Recently, we have shown that some of the different binding sites on CaM are allosterically related (Johnson, 1983; Mills & Johnson, 1985). This has led to the suggestion that the binding of endogenous regulators, either parts of target proteins or small molecules, to CaM may direct it to activate certain proteins specifically via these allosteric mechanisms. Studies concerning

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[1] Abbreviations: ANS, 8-anilinonaphthalenesulfonate; CaM, calmodulin; felodipine, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester; R24571, calmidazolium; TNS, 2-*p*-toluidinylnaphthalenesulfonate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; prenylamine, *N*-[2-(phenylmethyl)-ethyl]-3,3-diphenylpropylamine; BAPNA, benzoylarginine *p*-nitroanilide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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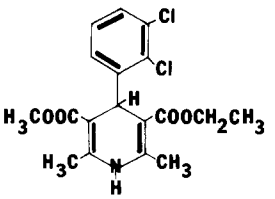

FIGURE 1: Chemical structure of felodipine.

the binding of the dihydropyridine calcium antagonist felodipine (Figure 1) have been particularly insightful in this respect. It binds to calmodulin in a Ca^{2+}-dependent manner with micromolar affinity (Boström et al., 1981; Johnson & Wittenauer, 1983). Its affinity for calmodulin can be increased by as much as 20-fold through an allosteric mechanism by the binding of drugs to other distinct sites on CaM (Johnson, 1983; Mills & Johnson, 1985).

In this study, we have examined various proteolytic fragments of CaM for their ability to bind felodipine in a Ca^{2+}-dependent manner. This approach has allowed us to localize a Ca^{2+}-dependent felodipine binding site between residues 1 and 90. Further, no evidence for allosteric interactions was found in any of these fragments.

Materials and Methods

Bovine testis CaM and its proteolytic fragments were prepared as described earlier (Andersson et al., 1983a; Vogel et al., 1983; Thulin et al., 1984). Both TR_1C and TR_2C are obtained after limited digestion of CaM with trypsin (100:1) for 6 min at room temperature. TM_1 and TM_2 are obtained by digestion with thrombin in the presence of Ca^{2+}. TR_2E is the major product when CaM is digested with trypsin (200:1) for 30 min at room temperature. It can be purified from smaller contaminating peptides by using gel exclusion chromatography as described earlier for the TM fragments (Andersson et al., 1983a). The purity of all fragments was confirmed by migration of the peptide as a single band on both SDS–polyacrylamide and agarose electrophoresis. The nature of the different peptides had been established earlier by partial amino acid sequencing, amino acid composition determinations, high-resolution proton NMR, and UV spectroscopy [see Andersson et al. (1983a), Vogel et al. (1983), and Thulin et al. (1984)]. The purified and characterized fragments and intact CaM were then used to calibrate the elution patterns for the different peptides on phenyl-Sepharose as well as on the FPLC pro-RPC column (see below).

Klee et al. (1981) and later Newton et al. (1984) reported on HPLC separation of proteolytic fragments of calmodulin using a Waters μBondapak alkylphenyl reverse-phase column. We have used a similar strategy and the same buffers as these workers, but we have employed Pharmacia FPLC equipment and a Pharmacia analytical pro-RPC HR 5/2 C8 substituted silica column to analyze the mixtures of fragments and CaM. The absorbance of the column eluent was measured at 215 mn, and the curves were automatically integrated. Using a very shallow gradient from 35 to 50% buffer B (which corresponds to 17.5–25% CH_3CN), we could separate TM_2, TR_2C, TR_1C, TM_1, and CaM without difficulties.[2] The separation of the peaks for TM_1 and TRC was rather poor, so it was difficult to be certain about these two fragments. However, the conversion of CaM to TR_2C was always almost quantitative, and thus, it can be concluded that no TM_1 was produced, only TR_1C. In the experiments reported here, al-

[2] S. Rochester, E. Thulin, and H. J. Vogel, unpublished results.

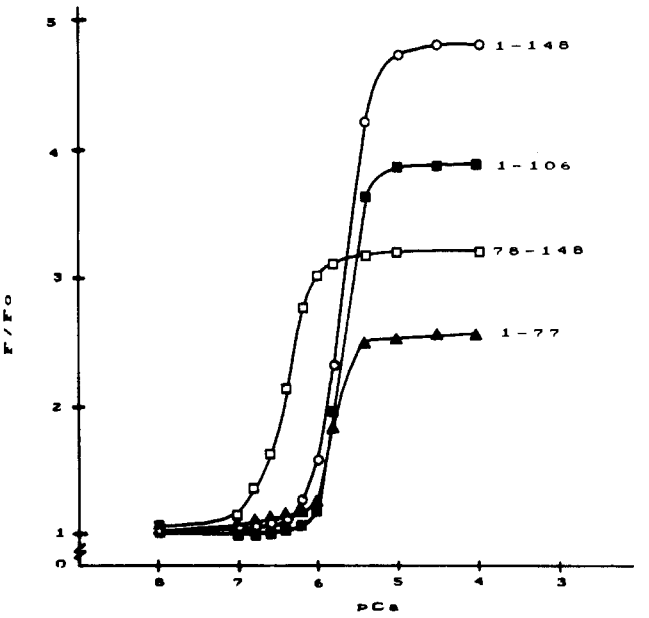

FIGURE 2: Calcium dependence of the interaction of TNS with CaM and its fragments. Calcium titrations of 4 μM TNS with 2 μM protein: CaM (○), fragment TR_1C (1–77) (▲), fragment TR_2C (78–148) (□), and fragment TM_1 (1–106) (■). Fragment TR_2E (1–90) gave a 4-fold fluorescence increase that was half-maximal at pCa 5.8, very similar to the response of fragment TM_1 (1–106) with TNS (data not shown). Titrations were conducted in 10 mM Mops, 90 mM KCl, and 2 mM EGTA maintained at pH 7.0 with KOH. Free calcium levels were determined by using known H^+ and Ca^{2+} binding constants for EGTA, as previously described (Johnson et al., 1978). Excitation was at 320 nm, and the emission was monitored at 470 nm.

though some CaM antagonists altered the rate of tryptic cleavage, the products of this cleavage (TR_1C and TR_2C) were unchanged. Thus, the binding of the drugs to CaM does not seem to alter the tryptic specificity for cleavage at Lys-77 on intact CaM.

Control experiments of the tryptic digestion of the model peptide BAPNA were performed following the absorbance of the released *p*-nitroaniline at 405 nm in the following mixture: 75 mM Tris, pH 7.8, and 1.50 mM $CaCl_2$, containing 0.015 mg/mL trypsin and varying amount of drug. Under these conditions linear curves for the *p*-nitroaniline release were obtained for more than 5 min, and no drug effects on trypsin activity were observed. Drugs and other spectroscopic probe molecules were obtained from the same sources as described earlier (Johnson & Wittenauer, 1983; Andersson et al., 1983b). All other chemicals were of analytical grade. Fluorescence measurements were performed as described earlier (Johnson, 1983).

Results

Localization of Hydrophobic Sites. Nonspecific hydrophobic fluorescent probes, such as ANS and TNS, have been found to bind with millimolar affinity to hydrophobic sites on CaM and undergo large fluorescence increases (LaPorte et al., 1980; Tanaka & Hidaka, 1980; Follenius & Gerard, 1984). They thus provide a means of detecting and characterizing the Ca^{2+}-dependent exposure of hydrophobic sites on CaM and its fragments. The Ca^{2+} dependence of TNS fluorescence in the presence of CaM and some of its proteolytic fragments is shown in Figure 2. Each fragment exhibited a Ca^{2+}-dependent fluorescence increase that was less than that seen with intact CaM. The sum of the increase in TNS fluorescence produced by its binding to TR_1C (1–77) (the amino-terminal half) and TR_2C (78–148) (the carboxy-terminal half) was approximately equal to the increase produced by TNS binding

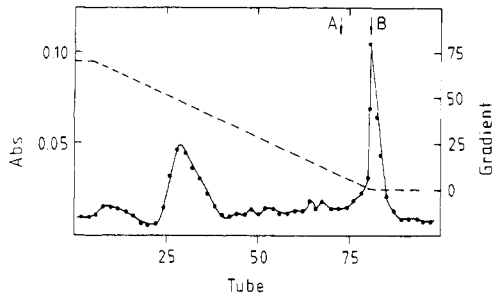

FIGURE 3: Chromatography of a mixture of TR_1C (1–77) and TR_2C (78–1400) on phenyl-Sepharose. The conditions have been described earlier (Vogel et al., 1983). The reversed Tris gradient was formed by mixing 75 mM Tris-HCl, pH 7.5, containing 0.5 mM $CaCl_2$ and 0.5 mM $CaCl_2$, pH 7.5. A similar profile was used when a sample was dialyzed into 100 mM KCl and a reversed KCl gradient was run. The absorbance was measured at 258 nm. At arrow A, a 1 mM EDTA solution was added. Arrow B indicates the elution position of native CaM under these conditions. The nature of TR_2C and TR_1C was confirmed by SDS-polyacrylamide and agarose gel electrophoresis. In addition, pure TR_2C and TR_1C fragments ran similarly to the two peptides in the mixture as shown in this figure.

to the intact protein. Furthermore, half-maximal binding occurred at pCa 6.4 for TR_2C (78–148) but at significantly higher [Ca^{2+}], pCa 5.8, for TR_1C (1–77). Fragments TM_1 (1–106) (Figure 1) and TR_2E (1–90) (data not shown) undergo 75% of the fluorescence change observed with intact CaM with essentially the same calcium dependence (half-maximal binding pCa 5.7–5.8) for each. The inclusion of residues 78–90 can therefore nearly double the fluorescence increase observed with 1–77 alone without producing a large change in the Ca^{2+} dependence. Fragment TM_2 (108–148) shows essentially no Ca^{2+}-dependent alteration in TNS fluorescence (data not shown).

Chromatography of CaM fragments on phenyl-Sepharose (Vogel et al., 1983; Brzeksa et al., 1983) as well as photoaffinity labeling studies (Krebs et al., 1984) has provided quite conclusive evidence that Ca^{2+}-exposed hydrophobic surfaces are located in both halves of CaM. Figure 3 shows the profile that is obtained when a linear reversed salt gradient is applied to a mixture of TR_1C (1–77) and TR_2C (78–148) that is applied to a phenyl-Sepharose column. TR_2C elutes around 50 mM KCl; TR_1C does not elute until EDTA is included (Figure 3, arrow A) in the distilled H_2O used to wash the column. In this regard, the TR_1C elution profile is more similar to that of intact CaM (Figure 3, arrow B). This experiment suggests that the most hydrophobic Ca^{2+}-exposed site is located in the amino-terminal half of the molecule, consistent with earlier experiments (Drabikowski & Brzeksa, 1983; Steiner, 1984).

Localization of Felodipine Site. When felodipine binds to intact CaM, it undergoes a large fluorescence increase that allows us to follow its Ca^{2+}-dependent binding (Figure 4A). Rather surprisingly, fragments TR_1C (1–77) and TR_2C (78–148), which each bind TNS in a Ca^{2+}-dependent manner, showed no Ca^{2+}-dependent enhancement of felodipine fluorescence. Further, TM_2 (108–148) showed no fluorescence enhancement with felodipine, similar to our results with this fragment and TNS. Fragment TM_1 (1–106), on the other hand, exhibited a Ca^{2+}-dependent felodipine fluorescence that was 67% of that observed with intact CaM. Fragment TR_2E (1–90) exhibited no significant increase in felodipine fluorescence either in the presence or in the absence of calcium.

While felodipine binding may not always be accompanied by an increase in felodipine fluorescence, its binding should always result in an increase in its polarization relative to free unbound felodipine. Figure 4B demonstrates the increase in

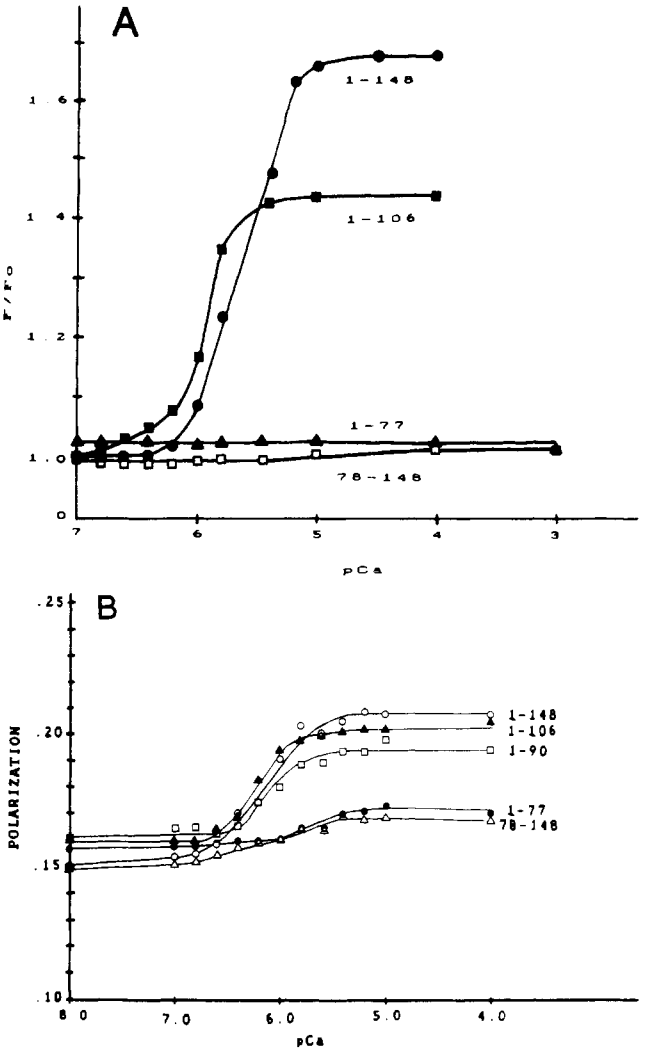

FIGURE 4: (A) The calcium dependence of the fluorescence of felodipine with CaM and its fragments. Calcium titrations of 4 μM felodipine and 2 μM CaM (●), fragment TM_1 (1–106) (■), fragment TR_1C (1–77) (▲), and fragment TR_2C (78–148) (□) are shown. Fragments TR_2E (1–90) and TM_2 (107–148) produced no calcium-dependent change in felodipine fluorescence. Titrations were conducted as described in Figure 1 with excitation and emission wavelengths at 365 and 445 nm, respectively. (B) Calcium dependence of felodipine fluorescence polarization in the presence of CaM and its fragments. Corrected polarization values are shown as a function of calcium for felodipine with whole CaM (○), fragment TR_1C (1–77) (●), fragment TR_2C (78–148) (△), fragment TM_1 (1–106) (▲), and fragment TR_2E (1–90) (□). Calcium titrations were conducted on 1 μM felodipine and 2 μM protein as described in Figure 2. Fluorescence polarization measurements were conducted as previously described (Cardin et al., 1982). Excitation was at 365 nm, and emission was monitored at 445 nm. Each curve represents the average of at least three experiments, and the standard deviation at each point was less than 11%.

the polarization of felodipine as it binds to CaM and its fragments as a function of calcium. Using this methodology, we once again see little binding of felodipine to TR_1C (1–77) or TR_2C (78–148). Each of these fragments exhibit only about 20% of the calcium-induced change in polarization that is observed with intact calmodulin. Thus, if felodipine is binding to either of these fragments, it is with very low affinity. Felodipine is found to bind to native CaM, fragment TM_1 (1–106), and fragment TR_2E (1–90) in a Ca^{2+}-dependent manner, producing ~75% and 64% of the change in polarization observed with intact calmodulin, respectively.

Further evidence for selectivity in felodipine binding come from studies of the effect of CaM antagonists and felodipine on the tryptic cleavage between residues 77 and 78.[3] There

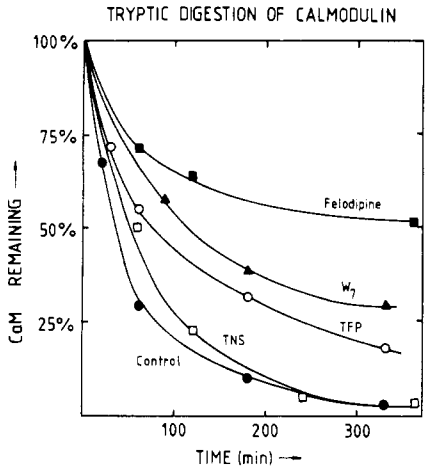

FIGURE 5: Tryptic cleavage of CaM in the presence and absence of calcium and CaM antagonists. A typical sample contained 2 mg of calmodulin, 0.25 mM drug, and 20 μg of trypsin in 1 mL. The reaction was stopped at the appropriate time by the addition of 2 equiv of soybean trypsin inhibitor (Andersson et al., 1983a). Samples were frozen at −20 °C until they were analyzed by FPLC as described under Materials and Methods. The appropriate amounts of the calcium and CaM antagonists were added from stock solutions in distilled H_2O or ethanol. Ethanol itself has little effect on the degradation rate of CaM by trypsin. Addition of TNS, ethanol, TFP, or felodipine in concentrations similar to those used in this experiments to trypsin did not effect the rate of tryptic degradation of BAPNA, thus excluding the possibility that the binding of these agents to trypsin changes the rate of proteolysis.

is reasonably good evidence that CaM possesses two binding domains for substances, such as TNS (Follenius & Gerard, 1984), TFP (Newton et al., 1983; Andersson et al., 1983b; Thulin et al., 1984; Vogel et al., 1984), and felodipine (Mills & Johnson, 1985). Hence, all experiments were performed in the presence of 2 equiv of drug. Figure 5 shows that felodipine offers significantly more protection against tryptic cleavage between residues 77 and 78 than any of the other compounds tested. Significantly, TNS offered no protection, whereas TFP and W7 were about half as effective as felodipine.

Lack of Allosteric Interactions in the Felodipine Binding Fragment. Previously, we have shown that the binding of prenylamine ($K_d \sim 2.4$ μM; Johnson & Wittenauer, 1983) to CaM can act via an allosteric mechanism to enhance felodipine binding, resulting in the fluorescence increase shown in Figure 6. In the absence of prenylamine, felodipine binds to calmodulin with a K_d of 8 μM. In the presence of 10 μM prenylamine, the affinity of calmodulin for felodipine is increased ~20-fold to $K_d \sim 0.4$ μM (Mills & Johnson, 1985). At higher concentrations, prenylamne will competitively displace felodipine, resulting in the observed decrease in fluorescence (Figure 6). The Hill coefficient for the prenylamine-induced fluorescence increase is 1.1 ($r = 0.99$), and for the fluorescence decrease, it is 1.04 ($r = 0.99$) with intact CaM. The effect of prenylamine on fragment TM_1 (1–106)–felodipine in the presence of Ca^{2+} is shown in Figure 6. Clearly, prenylamine produces no enhancement in TM_1 (1–106)–felodipine fluorescence but only a fluorescence decrease (Hill coefficient = 1.07, $r = 0.99$) similar to that which occurs when prenylamine competitively displaces felodipine from

[3] In fact, the tryptic proteolysis in the presence of Ca^{2+} is slightly more complicated. Not only the bond between residues 77 and 78 can be attacked but also those between residues 74 and 75 and 75 and 76 (Thulin et al., 1984; Newton et al., 1984). Nevertheless, cleavage between residues 77 and 78 is the most effective, and in all three cases fragments of approximately similar sizes are obtained.

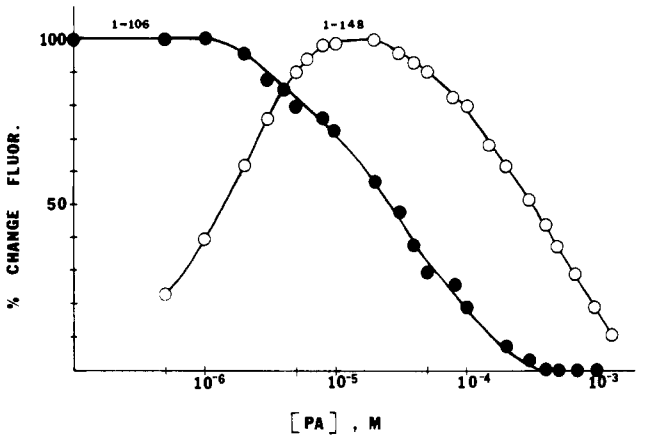

FIGURE 6: Prenylamine titrations of felodipine bound to calmodulin and felodipine bound to fragment TM_1 (1–106). Two micromolar felodipine and 1 μM protein at pCa 3.0 in 10 mM Mops, 90 mM KCl, 2 mM EGTA, and 3 mM calcium, pH 7.0, were titrated with prenylamine. The prenylamine titration of felodipine–CaM produced a 2.7-fold fluorescence increase followed by a full reversal of this increase at higher prenylamine concentrations (○). The prenylamine titration of felodipine–fragment TM_1 (1–106) produced a 20% fluorescence decrease (●). These data represent the average of three experiments. Standard errors were less than 10% at each point.

intact CaM. This suggests that the allosteric interactions that occur between the prenylamine binding site and the felodipine binding site to produce the fluorescence increase observed in intact calmodulin do not occur in the felodipine binding fragment TM_1 (1–106).

Discussion

One of the most interesting features of the Ca^{2+}–CaM complex is that it can be cleaved proteolytically into "halves" containing residues 1–77 and 78–148. Each of these fragments maintains the structure as well as the ability to undergo the Ca^{2+}-dependent structural changes that it enjoyed in the intact molecule. In particular, the spectra of the apoprotein and Ca^{2+} protein as well as the spectra of the Ca^{2+}-induced changes in each half of the CaM as determined by UV and CD spectroscopy (Drabikowski et al., 1982) and by NMR (Andersson et al., 1983a; Aulagaugh et al., 1984; Dalgarno et al., 1984; Ikura et al., 1984; Thulin et al., 1984) sum to the respective changes observed in the intact molecule. Therefore, these fragments serve as good models for the behavior of each half of CaM in the intact protein.

There is very strong evidence that Ca^{2+}-dependent hydrophobic binding domains exist in both TR_1C (1–77) and TR_2C (78–148) (Vogel et al., 1983; Brzeska et al., 1983; Krebs et al., 1984). Since CaM contains two binding sites for TNS (Follenius & Gerard, 1984), one would predict that each half of CaM would encompass one high-affinity TNS binding site. The studies reported here (Figure 2) indicate that this is indeed the case and that both fragments bind the fluorescent probe molecule TNS in a Ca^{2+}-dependent manner. Moreover, the sum of the changes observed in the fragments is very similar to the changes observed with TNS interactions with whole CaM, suggesting that the two TNS binding sites have not been dramatically affected by the proteolysis. Consistent with this notion is our observation that TNS does not protect against proteolytic attack in the region of residues 77–78 (see Figure 5). It is unlikely, therefore, that the TNS binding regions extend into this part of the protein. Fragment TR_2C (78–148) appears to exhibit the Ca^{2+}-dependent TNS binding at lower calcium concentrations than fragment TR_1C (1–77). This is consistent with the finding that TR_2C has a slightly higher apparent calcium affinity than TR_1C (Drabikowski et al.,

1982; Andersson et al., 1983a).

Phenyl-Sepharose chromatography indicates that the hydrophobic interactions are stonger in TR_1C than in TR_2C (see Figure 3). This would suggest that calmodulin antagonists that bind primarily through hydrophobic interactions might bind preferentially to the more hydrophobic site(s) in TR_1C (1–77). Cadmium-113 NMR studies of TFP binding to CaM and its proteolytic fragments (Andersson et al., 1983b; Thulin et al., 1984) suggest that the highest affinity site for TFP is located on the carboxy-terminal half. Thus, forces other than hydrophobicity alone must play a role in directing the binding of some charged calmodulin antagonists. It is likely that ionic interactions between the positively charged moieties on antagonists, such as TFP, prenylamine, and R24571, and negatively charged residues on the protein are important in directing binding. The three glutamic acid residues at positions 82, 83, and 84 seem logical candidates for this type of interaction.

Our fluorescence data (Figure 4) indicate some selectively in felodipine binding to CaM fragments. Under our experimental conditions, felodipine (unlike TNS) does not recognize the hydrophobic sites on TR_1C or TR_2C that are formed upon Ca^{2+} binding. It will bind, however, to the fragment that contains residues 1–90 (TR_2E) and to fragment TM_1 (1–106). Moreover, our proteolysis experiments (Figure 5) suggest that felodipine offers more protection against tryptic cleavage between residues 77 and 78 than the other CaM antagonists. All these data suggest that the inclusion of residues 78–90 is a prerequisite for proper felodipine binding. Presumably, the residues in this region are important for felodipine binding to the hydrophobic region existing in 1–77. The residues in region 78–90 are apparently not helpful in facilitating felodipine binding to the hydrodrophobic site(s) existing in 78–148, since this fragment does not bind felodipine. These studies suggest that felodipine binding to CaM is more specific than the binding of hydrophobic probe molecules (ANS, TNS, TFP, and phenyl groups). Further, these studies suggest that the primary felodipine binding site exists in the region 1–90 and that some of the residues of 78–90 are essential for felodipine binding.

The three-dimensional structure of calmodulin (Babu et al., 1985) demonstrates that, in the presence of calcium, residues 65–92 form a long central helix that is rather hydrophilic in nature. They suggest that this central helical region may be exposed with calcium binding to calmodulin. This is consistent with trypsin cutting at the center of this helix only in the presence of calcium. While this rather hydrophilic central helix may not provide the calcium-induced hydrophobic pockets for the binding of hydrophobic probe molecules and drugs, its polar residues may play an important role in providing the electrostatic interaction necessary for the high-affinity binding of some drugs with calmodulin. In fact, our results suggest that residues 78–90 in this helix are required for felodipine binding and that felodipine binding can protect from proteolytic attack in this region. The integrity of this central helix seems to be less important for TNS (and ANS) binding since they readily bind to both 1–77 and 78–148 in which the helix has been cut.

Malencik and Anderson (1984) have performed detailed studies on the binding of peptides to calmodulin. Their conclusion is that the major binding site for the high-affinity binding of mastoparans and melittin is located between residues 72–106. This is the same part of the molecule that is prerequisite for felodipine binding. Since we have found that melittin displaces felodipine directly from its allosterically potentiated specific binding site on calmodulin and moreover that it induces very similar changes in cadmium-113 NMR spectra of CaM as felodipine, it is likely that these two widely different compounds compete for the same site on CaM.[4]

In intact CaM, we have shown the existence of two felodipine binding sites that are allosterically related. Other CaM antagonists, including prenylamine and R24571, initially bind to one of these two felodipine binding sites and dramatically enhance felodipine binding (20-fold increase in affinity) to its remaining site. At higher concentrations, these CaM antagonists bind and displace felodipine from its potentiated site also, resulting in a fluorescence decrease [see Johnson (1983) and Mills & Johnson (1985)]. These effects are seen in Figure 6, where low concentrations of prenylamine lead to an increase and higher concentrations lead to a decrease for the CaM–felodipine fluorescence, both with a Hill coefficient of 1.0. Our studies on the effect of prenylamine on felodipine bound to the TM_1 (1–106) fragment indicate that these allosteric interactions do not occur in the fragment as they do in intact CaM. In the fragment, prenylamine simply competitively displaces felodipine from a single site, producing the observed fluorescence decrease. This suggests that the site where prenylamine and R24571 bind and potentiate felodipine binding in intact calmodulin does not exist in fragment 1–106.

Since each half of CaM (1–77 and 78–148) contains a hydrophobic ligand binding site, one interpretation of our data is that these two hydrophobic sites on opposite ends of the molecule are the allosterically related drug binding sites. Our fragment studies suggest that a felodipine site exists in fragment 1–90. It is, therefore, possible that felodipine binding to this site is potentiated by prenylamine or R24571 binding to the hydrophobic site that exists in the carboxy terminal half of the protein (residues 78–148).

Evidence for these allosteric mechanisms among other drug binding sites on calmodulin has recently been presented. Newton et al. (1983) have shown that low concentrations of TFP promote the binding of phenothiazine analogue whose positive charge has been replaced by an uncharged group. Under these conditions, TFP might bind to its site on the carboxy-terminal half of the molecule (Thulin et al., 1984), and the uncharged analogue would tend to exhibit a preference for the more hydrophobic site in the amino-terminal fragment. The carboxy-terminal TFP site is retained in the CaM fragment 77–123 (Head et al., 1982). As indicated earlier, inspection of this part of the protein molecule suggests that one or more of the Glu residues 82, 83, and 84 are ideally suited for neutralizing the single positive charge on TFP. Since these three residues are part of the central α-helix that connects the two halves of the CaM protein (Babu et al., 1984), one could envision that drug binding to the carboxy-terminal half would affect the structure of the connecting helix as well as the structure around the hydrophobic site on the amino-terminal half of CaM. Further research will be necessary to demonstrate conclusively if this suggested molecular mechanism forms the basis of the observed allosteric interactions between the different binding domains of CaM.

Registry No. TFP, 117-89-5; TNS, 7724-15-4; W7, 65595-90-6; Ca, 7440-70-2; felodipine, 72509-76-3; prenylamine, 390-64-7.

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pH-Dependent Lysis of Liposomes by Adenovirus

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ABSTRACT: Purified adenovirus induced a dose-dependent release of the water-soluble markers calcein and carboxyfluorescein from liposomes. Marker release was strongly dependent on pH, and at temperatures below 5 °C, the rate of release showed an optimum at a pH of about 6. This pH dependence parallels disruption of endocytic vesicles by adenovirus and the permeabilization that adenovirus induces on the cell surface. There did not seem to be a striking dependence on the lipid composition of the liposomes. Electron microscopy using a negative stain shows liposomes bound to adenovirus. In some cases, the liposomes were still intact, but many liposomes, which were attached to the vertices of the virus, appeared lysed. These data support the notion that adenovirus, which enters the host cell by receptor-mediated endocytosis, gains access to the cytoplasm by a subsequent pH-dependent disruption of the membrane of the endocytic vesicle.

Adenovirus is a nonenveloped virus whose nucleocapsid finds its way to the cytoplasm of the host cell by receptor-mediated endocytosis, followed by disruption of the the membrane of the endocytic vesicle (Dales, 1973; FitzGerald et al., 1983). This disruption is pH dependent and is abolished by treating cells with compounds that raise the pH of the endocytic vesicle (Seth et al., 1984a). The disrupting activity can be shown to take place directly on the plasma membrane, if cells with adenovirus attached to their surface receptors are placed at a decreased pH (Seth et al., 1984c).

In order to examine the mechanism of this membrane disruption, we studied the interaction of adenovirus with liposomes. In the present study, we show that purified adenovirus induces a rapid, pH-dependent release of the water-soluble markers calcein and carboxyfluorescein from liposomes. Electron micrographs suggest attachment of liposomes to the