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BINDING OF A DIHYDROPYRIDINE FELODIPINE-ANALOGUE TO CALMODULIN AND RELATED CALCIUM-BINDING PROTEINS

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Abstract—A dihydropyridine-affinity column was prepared by coupling a physiologically active and vasoselective amino-derivative of felodipine to divinylsulfone-activated Trisacryl GF2000. Calmodulin (CaM) as well as the homologous calcium-binding proteins skeletal and cardiac Troponin C (sTnC and cTnC) and S100b bound to this resin in a calcium-dependent manner. In contrast, other homologous proteins such as parvalbumin and the intestinal calcium-binding protein did not bind. Competition studies showed that CaM had a higher affinity for the felodipine-column than sTnC or cTnC. Through studies with a series of proteolytic fragments of CaM and sTnC, it was found that the felodipine binding site is located in the amino-terminal domain of the protein. These results illustrate the utility of affinity-chromatography for the study of dihydropyridine-binding proteins.

Calmodulin (CaM)‖ is a ubiquitous acidic heat-stable protein which is found in significant quantities in all eukaryotic cells [1]. The levels of CaM are particularly high in smooth muscle where the protein mediates the calcium-triggering of the contraction [2]. This represents only one of many-fold actions of CaM. At least twenty other enzyme and protein systems can be modulated by CaM in a calcium-dependent manner [1, 3]. CaM binds a total of four calcium ions in a specific sequence [4, 5]. The result of the Ca^{2+}-binding is that the protein undergoes a conformational change which exposes hydrophobic surfaces [4–9] that allow it to interact noncovalently with its target proteins and enzymes. Interestingly, various classes of drugs such as the phenothiazines [18], naphthalene-sulfone amides [11] and also various calcium-antagonists including the dihydropyridines [12, 13] can bind to CaM by combining with these surfaces (for review see Ref. 5). In doing so, they inhibit a variety of the regulatory actions of CaM [14, 15], and thus they display a calmodulin-antagonist behaviour. Although it is unlikely that there is a pharmacological relevance to all these interactions (for discussion see Ref. 14, for example), the delineation of the molecular interactions in the binding of clinically active drugs to CaM as a model protein may provide information about the nature of drug binding sites. Moreover, it has been shown that the calcium-channels—which appear to be the most important pharmacological target for the dihydropyridines—share a large number of properties with CaM, and they may therefore contain a protein domain which is likely to be homologous [4, 16].

Here we wish to report on a series of affinity chromatography studies using an immobilised dihydropyridine derivative. The use of this material has allowed us to study the interactions between felodipine and CaM, TnC, other calcium-binding proteins and a series of proteolytic fragments. CaM and sTnC have a very unusual dumbbell structure, which has been observed in the crystal [17, 18] and also in solution [19–23]. They are composed of two independently folded globular domains that are connected by an α-helical rod. Both the N- and C-terminal domains are capable of exposing a hydrophobic surface upon binding Ca^{2+} [8, 9], and they can each bind one equivalent of the phenothiazine TFP with high affinity [21]. Fluorescence studies have indicated that the binding of felodipine is different from that of the latter drug [24].

EXPERIMENTAL PROCEDURES

Materials. Calmodulin was purified from bovine testicles as described earlier [21]. Proteolytic fragments of CaM were prepared by limited proteolysis with trypsin (Sigma, TPCK-treated) and thrombin (Sigma). Tryptic fragmentation of Ca^{2+}-saturated CaM results in the production of both the N-terminal domain TR_1C (residues 1–77) and the C-terminal domain TR_2C (residues 78–148) [19, 21]. Thrombic cleavage performed in the presence of EDTA produces the fragments TM_1 (residues 1–106) and TM_2 (residues 107–148) [20]. The proteolytic cleavage scheme is schematically depicted in Fig. 1. The iden-

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‖ Abbreviations: CaM, calmodulin; TnC, Troponin C; sTnC, skeletal Troponin C; cTnC, cardiac Troponin C; NMR, nuclear magnetic resonance; TFP, trifluoperazine; and ICaBP, intestinal calcium-binding protein.

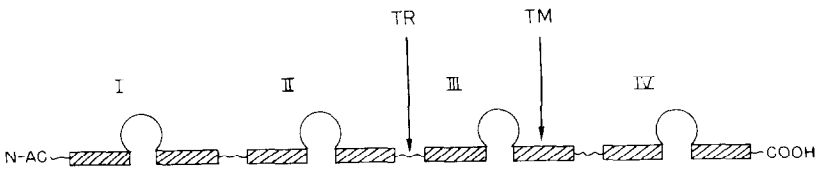

Fig. 1. Schematic diagram of calmodulin indicating the typical helix-Ca^{2+}-loop-helix pattern of the protein and the points of cleavage by trypsin (TR) and thrombin (TM).

tity and purity (>95%) of CaM and its proteolytic fragments were confirmed by sodium dodecyl sulfate (SDS)-gel electrophoresis and by high resolution proton NMR. Rabbit skeletal muscle Troponin C and beef heart cardiac muscle Troponin C were prepared according to Ref. 25 and they, together with bovine brain S100b [26] and carp parvalbumin [27], were a gift of E. Thulin, Department of Physical Chemistry, University of Lund, Lund, Sweden. The proteolytic fragments TR_1C (9–84) and TR_2C (89–157) of sTnC were prepared and characterized as described [22]. The porcine intestinal calcium-binding protein [28] was a gift from Dr. T. Hofmann, Department of Biochemistry, University of Toronto, Toronto, Canada. Bovine α-lactalbumin was purchased from Sigma. Trisacryl GF2000 was obtained from LKB, Sweden.

Synthesis of amino-felodipine. The amino derivative of felodipine was prepared as described below in two steps from 2-chloroethyl methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate (I) which is readily available through conventional Hantzsch synthesis. A mixture of 5.2 g (0.0124 mol) of I and 2.4 g (0.0370 mol) of sodium azide in 100 ml of dimethyl sulfoxide was heated with stirring at 80° for 18 hr, and then it was poured into 600 ml of ice-water. Extraction (ether), drying ($MgSO_4$), and evaporation gave an oily residue. Recrystallization from isopropranol gave 4.2 g of the expected product, 2-azidoethyl methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyri-dinedicarboxylate (II). The yield was 80%, and the melting point of II was 128–129°.

A solution of 4.2 g (0.0099 mol) of II and 7 ml of chloroform in 300 ml of ethanol was hydrogenated at atmospheric pressure using 1.0 g of 10% palladium on charcoal as a catalyst. Filtration and evaporation of the solvent gave amino-felodipine [2-aminoethyl methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate] in the form of the hydrochloride. Dissolving in water, neutralization, extraction with ether, drying ($MgSO_4$), and evaporation gave rise to the free base. Recrystallization of the crude product gave 2.6 g. The yield was 66% and the melting point of amino-felodipine, 72–76°. The structures of the compounds described above were confirmed by analytical and spectroscopic (^1H and ^{13}C NMR) data.

Coupling of amino-felodipine to Trisacryl GF2000. The coupling of amino-felodipine to divinylsulfone-activated Trisacryl GF2000 was carried out essentially as described in the instructions provided by the supplier (LKB). From the decrease in the amount of amino-felodipine in the reaction mixture after the coupling reaction, we determined the amount of amino-felodipine that was coupled to resin to be 50 μmol/ml.

Affinity chromatography. Affinity chromatography experiments were performed using a small column wrapped in aluminium foil containing 5 ml of the felodipine-affinity resin which was prepared as described in the previous section. The column was run at room temperature in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 100 mM KCl and either 5 mM $CaCl_2$ or 5 mM EDTA, pH 7.4. The A_{280} of the column eluate was measured to detect protein which did not bind to the felodipine-affinity column. Fractions were further checked for their protein contents by high resolution agarose gel electrophoresis [21, 29] and in some instances also by fast protein liquid chromatography (FPLC).

Determination of the pIC_{50}. The pIC_{50} values of felodipine and amino-felodipine were determined *in vitro* on the spontaneously active rat portal vein and the paced papillary muscle of the rat which were placed in the same organ bath [30]. The pIC_{50} is defined as the negative logarithm of the molar concentration (IC_{50} value) which would reduce by 50% the activity of these two preparations.

RESULTS

Design and testing of the affinity material. The structure of the highly vasoselective dihydropyridine-felodipine is shown in Fig. 2. Inspection of the structure revealed that it would be difficult to couple the drug directly to a solid support. Furthermore, it is well known that the potency and selectivity in the dihydropyridine series are very dependent on the substitution pattern of the two ring systems [31]. Consequently, we coupled felodipine to the resin via one of its ester side chains since structural modifications in that area of the molecule are usually better tolerated [31]. In addition, to reduce possible complications of steric hindrance we decided to use a relatively long spacer arm. These considerations led to our choice of the amino-felodipine-divinyl sulfone-Trisacryl GF2000 column. The structure of amino-felodipine is shown in Fig. 2, and its synthesis and coupling have been described in the Experimental Procedures. Before using the amino-felodipine column for the study of proteins, we determined the vasodilating potency and the negative inotropic myocardial potency of the drug analog. The results are listed in Table 1, where they are compared to the values determined for a typical selective and a non-vaso-selective calcium

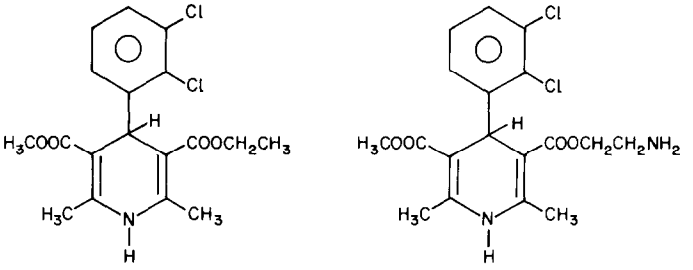

Fig. 2. Structures of felodipine and its amino-felodipine derivative.

antagonist. Obviously, the amino-felodipine had a reduced potency, but the compound had retained its vascular selectivity.

CaM only binds felodipine after addition of Ca^{2+} [12, 13]. Thus, a further crucial test for the validity of the affinity column procedure was to see if purified CaM would recognize the affinity ligand in a calcium-dependent manner. Figure 3 shows that fortunately this property was retained. CaM remained bound to the column only when Ca^{2+} was present.The maximal capacity of the column was determined at 1.1 mg CaM/ml of gel.

Table 1. Vascular and myocardial inhibitory potency (pIC_{50}) of amino-felodipine and other calcium antagonists*

Calcium antagonist	pIC_{50}	
	Portal vein	Papillary muscle
Felodipine†	7.47 ± 0.07	5.40 ± 0.13
Amino-felodipine	5.95 ± 0.10	4.49 ± 0.05
Verapamil†	6.60 ± 0.10	6.46 ± 0.08

* Values are means ± SEM; for felodipine and amino-felodipine, N = 6, and for verapamil, N = 7.
† Values were obtained from Ref. 30.

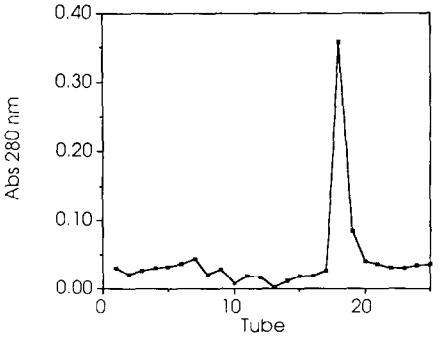

Fig. 3. Elution profile obtained with purified CaM on the felodipine affinity column. The protein (4 mg) was applied in the Ca^{2+}-containing buffer, and after tube 16 the EDTA containing buffer was applied.

* It also does not represent heterogeneity in these two protein fractions because when the protein that originally did not bind was reapplied to the same column a small fraction was retained, suggesting some form of heterogeneity in the column.

Binding of other calcium-binding proteins. CaM is a member of a group of calcium-binding proteins that share significant amino acid sequence homology and are therefore thought to originate from a common ancestor [1]. Thus, we checked the calcium-dependent binding of a series of homologous proteins to the felodipine affinity column. The results are listed in Table 2, and they show that paravalbumin and ICaBP did not bind, but that sTnC, cTnC and S100b all recognized felodipine in a calcium-dependent manner. The total binding capacity of the column for the latter three proteins approximated that for CaM (see Table 2). In these studies it is important not only to check for binding but to determine the total capacity of the column because it potentially allows one to differentiate between specific felodipine and other non-specific binding. For example, the maximum capacities obtained with sTnC, cTnC and S100b (Table 2) are consistent with the idea that there are an approximately equal amount of binding sites available on the matrix for these proteins. This suggests specific binding to the felodipine ligand. In contrast, the capacity of the felodipine-column for α-lactalbumin was very poor (<0.2 mg), thus suggesting that this does not reflect specific binding to the felodipine ligand but rather to a small non-specific hydrophobic portion of the column.* The calcium-binding protein α-lactalbumin was the only protein tested here that was not homologous to the other proteins and it should be noted that it bound to the column in reverse, i.e. it bound in the presence of EDTA and eluted upon addition

Table 2. Binding of calcium-binding proteins to the felodipine affinity column

Protein	Calcium-dependent binding	Maximum capacity (mg/ml)
Calmodulin	+	1.1
Skeletal muscle TnC	+	1.0
Cardiac muscle TnC	+	1.0
Bovine brain S100b	+	1.0
Parvalbumin	−	ND*
ICaBP	−	ND
α-Lactalbumin†	+	<0.2

* Not detectable.
† Calcium-dependent release.

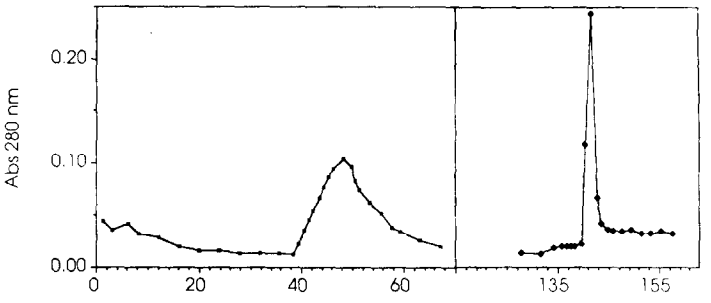

Fig. 4. Competition experiments between sTnC and CaM for binding to the felodipine-affinity column. For description see text.

Table 3. Binding of proteolytic fragments to the felodipine column

Proteolytic fragment	Ca^{2+}-dependent binding to felodipine column	Maximum capacity (mg/ml)	Binding to phenyl-Sepharose*
Calmodulin			
TR_1C (1–77)	+	>0.7	+
TR_2C (78–148)	–	<0.15	+
TM_1 (1–106)	–	<0.15	+
TM_2 (107–148)	–	ND†	–
Skeletal TnC			
TR_1C (1–77)	+	>0.7	+
TR_2C (78–148)	–	ND	+

* These results are shown to allow for a comparaison. The binding of these fragments to phenothiazine columns was identical to that observed with phenyl-Sepharose (data taken from Ref. 8).

† Not detectable.

of Ca^{2+}. It interacts with phenyl-Sepharose in an identical fashion [32].

Competitition between CaM and TnC. The data presented in Table 2 clearly establish that CaM and a few homologous calcium-binding proteins can expose a binding site for felodipine upon binding Ca^{2+}. As a next step we decided to determine which of the proteins had the highest affinity for felodipine. This question was addressed by experiments in which the felodipine-column was loaded to full capacity with sTnC (or cTnC). Subsequently a saturating load of CaM was applied. After an equilibration period of 5 min, the column was washed with the Ca^{2+}-containing buffer, and the amounts of CaM and sTnC were determined in all fractions by high resolution agarose gel electrophoresis and FPLC. The column profile obtained in this experiment is shown in Fig. 4. The broad peak that eluted first contained 80% TnC and 20% CaM, and the second peak that eluted with the EDTA-containing buffer contained over 85% CaM. Similar results were obtained with cTnC and in experiments where the column was first loaded with CaM and subsequently with sTnC (data not shown). From these results it appears that, of the three proteins tested, CaM has the highest affinity for felodipine.

Binding of proteolytic fragments of CaM and sTnC. To get an indication about the role of the two independently folded domains of CaM, we investigated whether a series of proteolytic CaM-fragments could bind in a calcium-dependent fashion to the felodipine affinity column. The data (Table 3) show that TR_1C (1–77) was the only fragment of CaM which displayed clear binding. Very small amounts of TR_2C (78–148) and TM_1 (1–106) bound too, but the maximal amount bound was so low and on the same order as observed for α-lactalbumin that it most likely represented the small non-specific hydrophobic part of the column and not any specific binding to felodipine.* Basically similar results were obtained with the two proteolytic fragments of sTnC (see Table 3). Again, the N-terminal domain appeared to contain the felodipine-binding site.

DISCUSSION

Several studies with felodipine have shown that it is a highly vasoselective agent with a high oral antihypertensive potency [33]. Thus, it is gratifying to note that the amino-felodipine which we have used here for the preparation of the affinity column displayed vasoselectivity which was similar to that of the parent compound, although its potency was slightly reduced (see Table 1). Consequently it would appear that the affinity-column provides a reasonable

* TR_2C fragments from CaM and sTnC are known to retain their native structures [21, 23], and thus the fact that it does not bind *cannot* be ascribed to the proteolytic fragment losing its correct structure.

means of studying the binding of felodipine to calcium-binding proteins. The data presented here show conclusively that not only CaM, but also its homologous proteins sTnC, cTnC and S100b can bind to felodipine after they have bound Ca^{2+}. This result is in agreement with fluorescence studies [16]. It is unlikely that all these interactions are of pharmacological significance. Nevertheless, despite the fact that the main action of felodipine appears to be on the calcium channels, some evidence for a second ary intracellular effect which could be mediated through CaM has been presented [14]. It should be noted that CaM, sTnC, cTnC and S100b are also capable of binding to phenyl-Sepharose and to a phenothiazine-affinity column [8, 34, 35]. In contrast, parvalbumin and ICaBP did not bind to these two affinity materials and, as we have demonstrated here, they also did not bind to the felodipine-affinity resin. Obviously the latter two proteins are not capable of exposing a hydrophobic surface upon binding Ca^{2+}, in contrast to the four earlier mentioned proteins. For CaM and TnC it is well-documented that the proteins undergo large conformational changes upon the binding or removal of Ca^{2+} [5]. This mode of operation is in keeping with their modulatory role where these two proteins translate an influx of Ca^{2+} into a contractile or metabolic response. In contrast, parvalbumins and ICaBP, which do not appear to modulate such events, do not undergo large changes in conformation [4] and thus it is unlikely that they would expose hydrophobic surfaces.

As the results obtained here with intact proteins are similar to those with phenyl-Sepharose and phenothiazine-Sepharose [8, 34], one may wonder if the exposure of a hydrophobic surface on these proteins is a sufficient condition for binding to any hyrophobic ligand or if there is some specificity? The data in Table 3 clearly show that the felodipine-affinity column provided specificity as the CaM TR_1C fragment but *not* the TR_2C fragment was retained. Both these fragments are known to bind to phenyl- and phenothiazine-Sepharose [8]. The TR_2C fragment which did not bind to the felodipine affinity column does, in fact, contain the strongest TFP binding site [21]. Consequently, the data presented here provide further evidence in support of the notion that the mode of felodipine binding is different from that of many other drugs and that one site appears to be localized in the N-terminal half [24].*

We were surprised that the fragment TM_1 did not show binding to the felodipine-column. In earlier fluorescence studies [24] and in a series of limited proteolysis studies (unpublished observations), we obtained experimental evidence suggesting the involvement of the α-helical rod that connects the two domains in the binding of felodipine. Based on these studies we would expect TM_1 to bind as well, or better than, TR_1C to an affinity column. Although it is known that both TR_2C (78–148) and TR_1C (1–77) retain their native structure, TM_2 (107–148) and the part of TM_1 (1–106) from 77 to 106 do not retain their native fold [4, 20–23]. Thus, it is likely that the denatured part of TM_1 blocks productive binding of this fragment under the conditions of the affinity chromatography experiment. Be that as it may, the dihydropyridine affinity column has displayed sufficient selectivity in these studies to conclude that it would be a useful tool for the purification of dihydropyridine-binding proteins in a manner similar to studies that have made use of phenothiazine columns [37]. In addition, the dihydropyridine column could be useful for the purification of calcium-channels [38]. Finally, it is worthwhile to note that the data obtained here and with two other affinity columns [8] are consistent with a recent report [39] in which it was found that there are at least three distinct sites for the binding of antangonists on calmodulin.

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* Mills *et al.* [36] have shown that calmodulin possesses two allosterically related felodipine binding sites. Our data clearly suggest that one of these is located in the N-terminal domain; the location of the second site is not clear. Although our data obtained with the felodipine affinity column are consistent with earlier fluorescence studies [24] and suggest that there is no binding site for felodipine on the C-terminal half, it is difficult to predict how the abolition of the allosteric interactions which occurs upon proteolytic fragmentation would affect the affinities.

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