Interactions of Amphiphilic Peptides Derived from \( \alpha_{\text{S2}} \)-Casein with Calmodulin

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ABSTRACT

Calmodulin-binding peptides, which had previously been isolated from a pepsin digest of \( \alpha \)-CN, were synthesized and then examined for their inhibitory effects on the activation of cyclic nucleotide phosphodiesterase that was induced by calmodulin. The concentrations of the synthetic peptides corresponding to 164–179, LKKISQRYQKFALPQY; 183–206, VYQHQMWAQPKTKVYPYR; and 183–207, VYQHQMWAQPKTKVYPYRVL, of \( \alpha_{\text{S2}} \)-CN that gave half-maximal inhibition were 65, 7.0, and 2.6 \( \mu \text{M} \), respectively. These inhibitory effects were reversed by increasing the amount of calmodulin. Fragments and analogs were prepared to study the interactions of the peptides with calmodulin in more detail. The results indicated that modification of the carboxyl terminus enhanced the affinities of the three peptides for calmodulin, and a region involved in the inhibition by \( \alpha_{\text{S2}} \)-CN (f183–207) was located at the carboxyl terminus 191–207. Two predicted calmodulin-binding sequences, 164–179 and 191–207 of \( \alpha_{\text{S2}} \)-CN, despite rather divergent primary structures, shared the structural motif common to the calmodulin-binding domains of the target proteins in the previously proposed complex model.

(Key words: casein, peptide-calmodulin interaction, complex model)

Abbreviation key: CaM = calmodulin, PDE = 3', 5'-cyclic nucleotide phosphodiesterase, IC\(_{50} \) = concentration giving half-maximal inhibition.

INTRODUCTION

O’Neill and DeGrado (17) reviewed research showing that a large number of positively charged amphiphilic peptide hormones and toxins bind to calmodulin (CaM) using a combination of hydrophobic and electrostatic interactions. Segments that are capable of adopting this structure are frequently found in the sequence of CaM-binding proteins. A preceding paper (12) showed that the positively charged C-terminal region of \( \alpha_{\text{S2}} \)-CN is an alternate source of CaM-binding peptides. The peptides corresponding to the sequences 164–179, LKKISQRYQKFALPQY; 183–206, VYQHQMWAQPKTKVYPYR; and 183–207, VYQHQMWAQPKTKVYPYRVL, of \( \alpha_{\text{S2}} \)-CN were isolated from a pepsin (EC 3.4.23.1) digest of \( \alpha \)-CN and inhibited CaM-induced phosphodiesterase (PDE; EC 3.1.4.17) activation. These three peptides shared the conspicuous feature that is common to the CaM-binding domain of the target enzymes and to certain neuropeptides that bind to CaM: a higher proportion of basic and hydrophobic residues.

Recently, the structures of the CaM-target peptide complexes in solution and crystalline form were resolved by nuclear magnetic resonance (10) and X-ray crystallography (15). Based on the structural data, a complex model was proposed, and its applications to the prediction of the complex formations between CaM and number of the known CaM-binding sequences were described (6, 10, 16). The present study investigated the interactions between CaM and the positively charged amphiphilic peptides derived from \( \alpha_{\text{S2}} \)-CN. For elucidation of the core sequences responsible for their binding properties to CaM and for interpretation of the manner of binding using the complex model, we chemically synthesized the CaM-binding peptides derived from \( \alpha_{\text{S2}} \)-CN according to the sequences and prepared their fragments and analogs from the synthetic peptides. These peptides were subjected to the investigation of the interaction with CaM to examine the correlation between the amino acid sequences and affinities for CaM.

MATERIALS AND METHODS

PDE Assay

Activity of PDE was measured in the absence or presence of CaM by the luciferin-luciferase (EC 1.13.12.7) technique (23), as previously described (12). Briefly, 0.1 mU of bovine brain PDE (Sigma Chemical Co., St. Louis, MO) was reacted at 37°C for 30 min in 150 \( \mu \text{L} \) of 50 mM glycylglycine buffer (pH 1996 J Dairy Sci 79:1728–1733 1728
8.0) containing 10 μg of pyruvate kinase (EC 2.7.1.40; Boehringer, Mannheim, Germany), 5 μg of myokinase (EC 2.7.4.3; Boehringer), 50 μg of cyclic AMP, 0.29 mg of ammonium acetate, 43 μg of MgCl₂, 6.5 μg of phosphoenolpyruvate, 0.4 mg of dithiothreitol, 15 μg of bovine serum albumin, 7.8 pg of GTP, and various concentrations of the peptides. The PDE assays conducted in the presence of CaM contained 3.3 pg of CaCl₂ and 0.2 U of bovine brain CaM (Sigma Chemical Co.) in the reaction mixture. In the absence of CaM, PDE assays were done with 0.11 mg of EGTA. When reversal of the peptide inhibition of PDE by CaM was investigated, 3.3 pg of CaCl₂ and various amounts of CaM were contained in the reaction mixture. After heat treatment at 100°C for 10 min, a portion of the mixture was added to the ATP assay kit (LKB Wallac, Turku, Finland), and light emission was recorded with a luminometer 1250 (LKB Wallac).

Peptide Concentration

Concentrations of peptides were determined at 280 nm by using the Tyr and Trp extinction coefficients of 1340 and 5500, respectively.

Syntheses of Peptides

Synthetic peptides were prepared on a solid-phase peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) using the standard cycle for Boc strategy. According to the sequence of the peptide, the Boc amino acid was activated with N,N'-dichlohexylcarbodiimide/l-hydroxybenzotriazole and then reacted with the deprotected amino terminal of growing peptide chain on a 4-(oxymethyl)-phenylacetoamidemethyl copoly(styrene-divinyl-benzen) resin. Simultaneous deprotection and cleavage of the as₂-CN (f164-179) from the resin were achieved with a procedure using trimethylsilylbromide, thioanisol, and trifluoroacetic acid (24). Simultaneous deprotection and cleavage of as₂-CN (f183-206) and as₂-CN (f183-207), peptides containing His and Trp, were achieved with a procedure using hydrogen fluoride (20) after deprotection treatment of 2,4'-dinitrophenyl group with thiophenol (19). The resulting residue was washed with dichloromethane and ether, and then the synthetic peptide was extracted with acetic acid and water (3:7, vol/vol). After purification using C₁₈ reversed-phase HPLC, the correct synthesis of each peptide was confirmed by amino acid composition and sequence analysis.

Fragmentation and Modification of CaM-Binding Peptide

The synthetic peptide was fragmented by enzymatic digestion and chemical cleavage. α₅₂-Casein (f189-207) and α₅₂-CN (f198-207) were prepared from α₅₂-CN (f183-207) by cleavage of the lysyl peptide bonds with lysyl endopeptidase (EC 3.4.21.50; Wako Pure Chemical, Osaka, Japan) (21). α₅₂-Casein (f191-207), which had been obtained during the course of the sequence determination of α₅₂-CN (4), was prepared from α₅₂-CN (f183-207) by cleavage of the methionyl peptide bond with cyanogenbromide (18). The peptides modified by the α-carboxyl group were readily prepared from the synthetic native peptides using the chemical methods because the carboxyl terminus was their only acidic group. The C-terminal methyl ester was prepared by condensation with MeOH in the presence of HCl (13). The C-terminus was amidated by condensation with NH₄Cl using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (14). Purification of the fragments and analogs was made using C₁₈ reversed-phase HPLC.

Electrophoresis

Electrophoresis of the mixture of Ca²⁺, CaM, and peptide was performed to examine the complex formation between CaM and peptides under nondenaturing conditions using 10% acrylamide gel (8, 9).

RESULTS

Effects of Synthetic Peptides on PDE Activation Induced by CaM

Figure 1 shows the effect of synthetic α₅₂-CN (f164-179), α₅₂-CN (f183-206), and α₅₂-CN (f183-207) on PDE activity in the presence and absence of CaM. The concentrations of the synthetic peptides giving half-maximal inhibition (IC₅₀) of PDE in the presence of CaM were approximately 65, 7.0, and 2.4 μM, respectively. These IC₅₀ values were essentially consistent with previously reported values for the isolated peptides from α-CN peptone (12). The concentration of each peptide that totally inhibited the activation of PDE induced by CaM had no significant effect on the basal PDE activity. The inhibitory actions of α₅₂-CN (f164-179), α₅₂-CN (f183-206), and α₅₂-CN (f183-207) were overcome by increasing the amount of CaM, as shown in Figure 2. These results indicate that the inhibition of PDE resulted from interactions between CaM and peptides derived from α₅₂-CN.
CaM-Binding Region of α_{s2}-CN (f183–207)

The amino acid sequences and the IC₅₀ values of fragments prepared from synthetic α_{s2}-CN (f183–207) are summarized in Table 1. The IC₅₀ values of α_{s2}-CN (f189–207) and α_{s2}-CN (f191–207) were within an order of the IC₅₀ value of α_{s2}-CN (f183–207); α_{s2}-CN (f198–207) did not affect PDE activity at the concentration up to 30 μM. Thus, the N-terminal region (8 residues) of α_{s2}-CN (f183–207) was not involved in the inhibition of the PDE activation that was induced by CaM. The difference between the IC₅₀ value of α_{s2}-CN (f183–206) and α_{s2}-CN (f183–207) demonstrated the contribution of the C-terminal residue to the potency. These results indicate that the CaM-binding site of α_{s2}-CN (f183–207) was located on the C-terminal region, and the potency was accounted for by the last 17 residues.

Effect of Carboxyl Terminal Modification in CaM Binding

The α_{s2}-CN (f164–179)-NH₂, α_{s2}-CN (f183–206)-NH₂, α_{s2}-CN (f183–207)-NH₂, and α_{s2}-CN (f183–207)-OCH₃ produced the same inhibitory effects at concentrations that were 7- to 34-fold lower than those of native peptides (Table 1). Complex formations between α_{s2}-CN (f183–207)-NH₂ and CaM in a gel electrophoresis under nondenaturing condition were shown in Figure 3. These peptide-CaM complexes have been reported with some CaM-binding peptides that have comparatively high affinities for CaM (7, 8, 9). These results indicate that amidation and methyl esterification of the C-terminus of the peptides enhanced affinities for CaM remarkably, and their IC₅₀ values were comparable with those of melittin (2) and vasoactive intestinal peptide (3).

Table 1. Effects of peptides derived from α_{s2}-CN on 3',5'-cyclic nucleotide phosphodiesterase (PDE) activation induced by calmodulin (CaM).

<table>
<thead>
<tr>
<th>Peptide from α_{s2}-CN</th>
<th>Sequence</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(f164–179)</td>
<td>LKKISQRYQKFALPQY</td>
<td>65</td>
</tr>
<tr>
<td>(f164–179) amid</td>
<td>LKKISQRYQKFALPQYNH₂</td>
<td>2.9</td>
</tr>
<tr>
<td>(f183–206)</td>
<td>VYQHQKAMKPWIQPKTVIPYVRY</td>
<td>7.0</td>
</tr>
<tr>
<td>(f183–206) amid</td>
<td>VYQHQKAMKPWIQPKTVIPYVRYNH₂</td>
<td>0.95</td>
</tr>
<tr>
<td>(f189–207)</td>
<td>AMKPWIQPKTVIPYVRY</td>
<td>2.8</td>
</tr>
<tr>
<td>(f191–207)</td>
<td>KPWIQPKTVIPYVRY</td>
<td>1.1</td>
</tr>
<tr>
<td>(f183–207) methyl ester</td>
<td>VYQHQKAMKPWIQPKTVIPYVRY-CH₃</td>
<td>0.14</td>
</tr>
<tr>
<td>(f183–207) amid</td>
<td>VYQHQKAMKPWIQPKTVIPYVRYNH₂</td>
<td>0.076</td>
</tr>
</tbody>
</table>

The concentration of peptide that reduced the PDE activation induced by CaM by 50%.

DISCUSSION

The present results demonstrated that the interactions between CaM and the amphiphilic peptides derived from α_{s2}-CN, which had previously been isolated from a pepsin digest of α-CN (12), caused the inhibition of PDE activation induced by CaM. Although the
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...hydrophobic residues at intervals of three or four residues. Although \( \alpha_{s2}-\text{CN} \) and C-terminal region of \( \alpha_{s2}-\text{CN} \) seemed to be atypical of CaM-binding sequences, such as those in melittin (7) and the CaM-binding domain of myristoylated Ala-rich protein related to C kinase substrate (22), because they contained Pro residues, the CaM-binding peptides might form helical formations in the complex. The enhancement of affinity for CaM with the modification of C-terminus (Table 1) might result from promoting a greater proportion of a helical conformation.

Based on the three-dimensional structure of CaM-target peptide complexes, as determined by nuclear magnetic resonance (10) and X-ray crystallography (15), a complex model was proposed (6, 10, 16). In the complex model, a major determinant of molecular recognition was the hydrophobic interactions between the hydrophobic residues of target peptides and the two shallow hydrophobic pockets in N- and C-terminal domains of CaM. The critical element in the hydrophobic interactions appeared to be the presence of two long-chain hydrophobic or aromatic residues in the CaM-binding sequence separated by 8 or 12 residues in order to anchor the peptide to the two...
domains of CaM (16). The complex model was adapted for the number of known CaM-binding sequences, including the peptide with the Pro residue such as melittin (6). Although complete elucidation of the precise details of the interaction of peptides that were derived from α-s2-CN and CaM requires further physical and chemical studies, we currently can speculate on the mode of the interaction using the complex model. On the basis of the complex model, predicted interactions of α-s2-CN (f164–179) and α,2-CN (f191–207) with CaM were shown in Figure 4. In α-s2-CN (f164–179), Ile-167 and Leu-176, which are located at positions separated by 8 residues, would anchor the peptide to the two domains of CaM. In addition, α-s2-CN (f164–179) conserved hydrophobic residues, Tyr-171 and Phe-174, at the positions corresponding to the C-terminal domain and both interacting domains of CaM, respectively. In the case of α-s2-CN (f191–207), Ile-194 and Leu-207, which are located at the positions separated by 12 residues, would be exclusively in contact with the C- and N-terminal domains of CaM, respectively. α-s2-Casein (f191–207) also conserved Thr-198, Ile-201, and Tyr-203 at the position that would interact with the C-terminal domain, both domains, and the N-terminal domain of CaM, respectively. The important contribution of Leu-207 is suggested by the difference between the IC50 values of α-s2-CN (f191–207) and α-s2-CN (f191–206). Recently, it has been proposed (1) that negatively charged margins at the binding surface extremities of CaM interact with basic peptide residues. The Lys-165 and Lys-166 of α-s2-CN (f164–179) and Lys-191 of α-s2-CN (f191–207) would favorably interact with the first negatively charged margin of CaM (e.g., Glu-7, 11, 14, 114, 120, 123, and 127). The remarkable enhancement of affinities for CaM by C-terminal modification suggested the putative unfavorable electrostatic interactions between the terminal carboxyl group of the native peptides and negative charges on the second acidic margin of CaM (e.g., Glu-83, Glu-84, and Glu-87).

In conclusion, the present study demonstrated that the effects of the peptides released from α-s2-CN by pepsin digestion on PDE activation induced by CaM were accounted for by the two CaM-binding sequences 164–179 and 191–207 of α-s2-CN. The two sequences, despite divergent primary structures, shared the structural motif common to the CaM-binding domains of the target proteins in the complex model, suggesting that the CaM-binding peptides derived from α-s2-CN interact with CaM similarly.

REFERENCES


Figure 4. Interactions of α-s2-CN (f164–179) and α,2-CN (f191–207) with calmodulin (CaM). Hydrophobic residues of CaM-binding peptides corresponding to the interacting positions on the N- and C-terminal domains of CaM are boxed. The ideal candidate target for CaM is a peptide with bulky hydrophobic residues at the negatively charged margins at the binding surface of CaM. The remarkable enhancement of affinities for CaM by C-terminal modification suggested the putative unfavorable electrostatic interactions between the terminal carboxyl group of the native peptides and negative charges on the second acidic margin of CaM (e.g., Glu-83, Glu-84, and Glu-87).