# Calmodulin Binding Peptide Comprising α-Casein Exorphin Sequence

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Although not homologous to any known calmodulin binding sequences, $\alpha_{s1}$-casein 90−109 (RYLGYLEQLLRLKKYKVPQL), the initial seven residues corresponding to α-casein exorphin sequence, seems to be endowed with the molecular feature characteristic of this class of peptides: a higher proportion of basic and hydrophobic residues. $\alpha_{s1}$-Casein 90−109 was synthesized, and its calmodulin binding was examined. $\alpha_{s1}$-Casein 90−109 reduced the calmodulin-induced cyclic nucleotide phosphodiesterase activation at the comparable concentration to that previously reported for endogenous opioid peptides such as *β*-endorphin and dynorphin. $\alpha_{s1}$-Casein 90−109 as well as the endogenous opioid peptides shares the common structural motif matching for the interacting domains of calmodulin in the previously proposed complex model, suggesting that these opioid peptides may interact with calmodulin in a similar manner.

**Keywords:** *Calmodulin; casein; exorphin; complex model; opioid peptide*

## INTRODUCTION

In addition to a nutritional role, casein has been recognized as a source of biologically active peptides (Fiat et al., 1993). Previously, we have isolated calmodulin (CaM) binding peptides from a pepsin hydrolysate of α-casein (Kizawa et al., 1995). The CaM binding peptides were released from the C-terminal basic and hydrophobic region of $\alpha_{s2}$-casein, and they shared the common structural motif apparent for the CaM binding sequence of the target enzymes (Kizawa et al., 1996). The first of numerous previously reported CaM binding peptides (O'Neil and DeGrado, 1990) was *β*-endorphin and dynorphin (Sellinger-Barnett and Weiss, 1982). These CaM binding opioid peptides, provided similar peptides were endowed with milk-constituting proteins, can afford a plausible account for certain physiological effects reported with milk (Smotherman and Robinson, 1993). From five to seven residue peptides with opioid activity, exorphins (Zioudrou et al., 1979; Loukas et al., 1983) and *β*-casomorphins (Henschen et al., 1979), have already been derived from casein hydrolysates. However, exorphins and *β*-casomorphins reported to date appear to be difficult to interact with CaM because most of the CaM binding peptides are about 20 amino acid residues long and their CaM binding motifs consist of an α-helix with a minimum of 12 residues (Erickson-Vitanen and DeGrado, 1987).

Examination of the amino acid sequence suggested that α-casein exorphin ($\alpha_{s1}$-casein 90−96) is located at the basic and hydrophobic region of $\alpha_{s1}$-casein. Although not homologous to any known CaM binding sequences, the sequence 90−109, RYLGYLEQLLRLKKYKVPQL, of $\alpha_{s1}$-casein seems to be endowed with a molecular feature characteristic of CaM binding peptides: a higher proportion of basic and frequently repeated hydrophobic residues. In this study, $\alpha_{s1}$-casein 90−109 was synthesized and its effect on CaM-induced 3′,5′-cyclic nucleotide phosphodiesterase (PDE) activation was examined.

## MATERIALS AND METHODS

$\alpha_{s1}$-Casein 90−109 and 90−96 were synthesized on a 431A solid-phase peptide synthesizer (Applied Biosystems) using the standard cycle for *tert*-butyloxycarbonyl strategy and dicyclohexylcarbodiimide/*N*-hydroxybenzotriazol activation. Simultaneous deprotection and cleavage of the peptide from the resin were achieved using trifluoromethansulfonic acid (Yajima et al., 1988). Purification was made using a preparative $C_{18}$ reverse-phase HPLC column R-355-15 (50 mm × 500 mm, YMC). Correct syntheses were confirmed by protonated molecule ion ($[M + H]^+$) analysis on a Kompact Maldi III time-of-flight mass spectrometer (Kratos Analytical) using a sinapinic acid matrix. Concentrations of synthetic peptides were determined at 275 nm by using the Tyr extinction coefficient of 1340.

PDE activity was measured by the luciferin−luciferase technique (Weiss et al., 1972) as previously described (Kizawa et al., 1995). Briefly, the CaM dependent PDE assays were done in 150 μL of 50 mM glycylglycine buffer (pH 8.0) containing 0.1 mU (U as defined by Sigma) of bovine brain PDE (Sigma), 10 μg of pyruvate kinase, 5 μg of myokinase, 50 μg of cyclic AMP, 0.29 mg ammonium acetate, 43 μg of $MgCl_2$, 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 with 3.3 μg of $CaCl_2$ and 0.2 U (U as defined by Sigma) of bovine brain CaM (Sigma) at 37 °C for 30 min, and the CaM independent PDE assays were done with 0.11 mg of EGTA. PDE activity was estimated through the contents of ATP, converted from hydrolyzed cAMP, in the reaction mixture.

## RESULTS AND DISCUSSION

The effects of the synthetic $\alpha_{s1}$-casein 90−109 and α-casein exorphin 90−96 on PDE activity are shown in Figure 1. $\alpha_{s1}$-Casein 90−109 inhibited CaM dependent PDE activity at a concentration lower than that previously reported for CaM binding peptides derived from $\alpha_{s2}$-casein (Kizawa et al., 1996). The concentration of $\alpha_{s1}$-casein 90−109 giving half-maximal inhibition for the activation of PDE by CaM ($IC_{50} = 1.0$ μM) is comparable to the reported values for certain opioid peptides (Sellinger-Barnette and Weiss, 1982) and slightly higher than that for the vasoactive intestinal peptide (Barnette and Weiss, 1985). $\alpha_{s1}$-Casein 90−109 did not affect the basal PDE activity at the concentration that totally inhibited CaM-induced PDE activation, indicating that the PDE inhibition was caused by the interaction between $\alpha_{s1}$-casein 90−109 and CaM. On the grounds that α-casein exorphin 90−96 (the seven N-terminal residues of $\alpha_{s1}$-casein 90−109) did not affect CaM-

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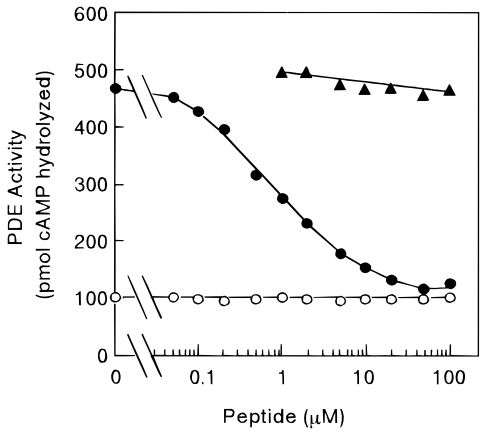


**Figure 1.** Effects of $\alpha_{s1}$-casein 90−109 and α-casein exorphin 90−96 on PDE activity. CaM dependent PDE activity were assayed in the presence of $\alpha_{s1}$-casein 90−109 (●) and α-casein exorphin 90−96 (▲). CaM independent PDE activity were assayed in the presence of $\alpha_{s1}$-casein 90−109 (○). The PDE activities are represented as the amounts of hydrolyzed cAMP for 30 min in one assay tube, and each point is the mean of duplicate determinations.

induced PDE activation at concentrations up to 100 μM as shown in Figure 1 and that $\alpha_{s1}$-casein 99−109 (the 11 C-terminal residues of $\alpha_{s1}$-casein 90−109) had been found in an inactive fraction of a pepsin hydrolysate of α-casein (unpublished data), almost the whole sequence of $\alpha_{s1}$-casein 90−109 is probably involved in the interaction with CaM.

Complex CaM-target peptide models have been proposed based on the structural data (Ikura et al., 1992; Meador et al., 1993), and these models were shown to be adaptable for the interactions between CaM binding peptides derived from $\alpha_{s2}$-casein and CaM (Kizawa et al., 1996). In the complex model, a major determinant in molecular recognition is the hydrophobic interactions between the shallow hydrophobic pockets in the C- and N-terminal interacting domains of CaM and the specific hydrophobic residues of target peptides, and the two hydrophobic residues in the CaM binding segment separated by 8 or 12 residues (i.e., +9 or +13 position) are the critical elements. Specifically, four hydrophobic pockets A ($Val^{144}$, $Met^{145}$, $Ala^{88}$, $Phe^{92}$, $Leu^{105}$, $Met^{124}$, $Ala^{128}$), B ($Ala^{88}$, $Leu^{39}$, $Leu^{112}$, $Phe^{92}$), C ($Leu^{112}$, $Leu^{39}$, $Met^{36}$, $Leu^{32}$, $Phe^{19}$), and D ($Leu^{32}$, $Met^{51}$, $Val^{55}$, $Leu^{71}$, $Phe^{68}$, $Phe^{19}$) of CaM can accommodate bulky hydrophobic residues at the positions (0, +4), (+7), (+9), and (+10, +13) of the target peptide, respectively (Afshar et al., 1994). The sequences of β-endorphin, dynorphin, and $\alpha_{s1}$-casein 90−109 are shown with the four hydrophobic pockets of CaM in Figure 2. Considering that the CaM binding domain of porcine β-endorphin is localized in the segment 14−25 (Giedroc et al. 1983), $Leu^{14}$ would occupy pocket A, and $Ile^{23}$ separated by eight residues, would occupy pocket C, and they would anchor β-endorphin to the C- and N-terminal interacting domains of CaM. Moreover, the residue $Phe^{18}$ and $Ala^{21}$ occupy pockets A and B of CaM, respectively. It is noteworthy that human β-endorphin, which replaces $His^{27}$ of bovine β-endorphin by $Tyr^{27}$, contains two hydrophobic residues separated by 12 residues, and $Tyr^{27}$ would occupy the pocket D. The removal of a basic residue and N-terminal pentapeptide from dynorphin resulted in a decrease of CaM binding (Malencik and Anderson, 1984), suggesting that almost the entire sequence of dynorphin is involved in the interaction with CaM. In the case of dynorphin, $Tyr^{1}$ would occupy

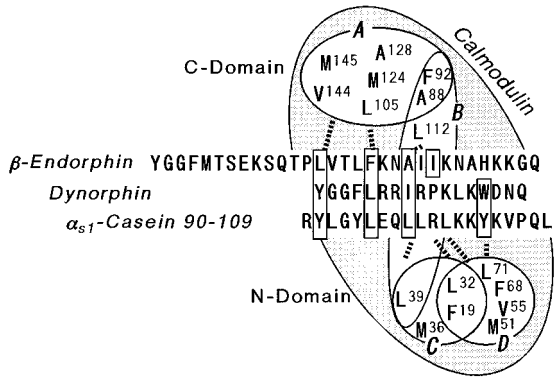


**Figure 2.** Interaction of β-endorphin, dynorphin, and $\alpha_{s1}$-casein 90−109 with CaM. Hydrophobic residues of the opioid peptides at the predicted positions corresponding to the four hydrophobic pockets (A−D) of CaM (Afshar et al., 1994) are boxed.

pocket A, and $Trp^{14}$, separated by 12 residues, would occupy pocket D, and they would anchor dynorphin to the C- and N-terminal domains of CaM. Moreover, residue $Leu^{5}$ and $Ile^{8}$ would occupy the pockets A and B of CaM, respectively. These adequate fittings indicate that the complex model is adaptable for the opioid peptides with moderate binding affinity for CaM. In the case of $\alpha_{s1}$-casein 90−109, $Tyr^{91}$ would occupy pocket A, and $Tyr^{104}$, separated by 12 residues, would occupy pocket D, and they would anchor $\alpha_{s1}$-casein 90−109 to the C- and N-interacting domains of CaM. Moreover, residue $Leu^{95}$ and $Leu^{98}$ occupy the pockets A and B of CaM, respectively. In the complex, the α-casein exorphin sequence comprised of $\alpha_{s1}$-casein 90−109 is eventually located at the same position with the leucine−enkephalin sequence of dynorphin. Results of the three-dimensional molecular modeling of $\alpha_{s1}$-casein (Kumosinski et al. 1991) predicted that there is an α-helical region in the sequence between $Tyr^{91}$ and $Tyr^{104}$. Thus, $\alpha_{s1}$-casein 90−109 seems capable of adopting a basic amphiphilic helix consistent with the structural motif requirements for the interacting domains of CaM.

The present study demonstrated the interaction of $\alpha_{s1}$-casein 90−109, comprising α-casein exorphin, with CaM. Its affinity for CaM and the predicted interacting manner seem to be similar to those of β-endorphin and dynorphin. The opioid receptor mediated physiological effects of the exorphin (Kil and Frestschel, 1994), casein (Defilippi et al., 1995), and milk (Blass and Fitzgerald, 1988; Smotherman and Robinson, 1993) have been reported. Further pharmacological studies will be necessary to elucidate the physiological roles of the CaM binding peptide derived from $\alpha_{s1}$-casein.

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