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Small Peptide Interacting with Pollen Calmodulin and their Effects on Cellular Functions

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Abstract: The interaction between dansyl-labeled pollen calmodulin (D-pCaM) and synthesized peptides was studied in the presence of Ca^{2+} by fluorescence spectra. It is found that Gly/L-Ala→D-Ala substitution in peptide chains caused great changes in their affinity for pCaM. Besides, our data provided evidence on the dissimilarity of different CaMs although they have highly-conserved structures. A preliminary study was carried out on the effects of CaM-binding peptides on cellular signal transduction, cell proliferation, showing the participation of CaM in cell functions mentioned above.

Keywords: Pollen calmodulin, affinity, cellular signal transduction, cell proliferation.

Calmodulin (CaM), a small Ca^{2+}-binding protein in all eukaryotic organisms, regulates many differentiated functions in cell through modulation of Ca^{2+} signals and interaction with some important enzymes. Although the amino acid sequences of calmlodulins from different species are highly conserved[1], there are some distinctions in their properties[2].

Up to now, animal CaMs have always been used to study the interaction of this protein with target peptides so as to find efficient CaM antagonists. In this report, the discovery of synthesized peptides' binding to rape pollen calmodulin (pCaM), a plant CaM which can obviously activate the bovine heart muscle PDE as the animal CaMs do[3], is presented in order to look for high-affinity antagonistic peptide for pCaM. We discussed structural factors that affect the peptide capacity of binding to pCaM, and analyzed similarity and difference between the interactions of peptides with pCaM and with bovine brain CaM (bCaM). Preliminary bioactivity tests for these peptides are also demonstrated.

Peptides described in **Table 1** were manually synthesized by a standard solid-phase method (DCC/HOBT) using Boc chemistry. MBHA resin was used to obtain C-terminal amides BP-13, 14 and 15. Final treatment in anhydrous liquid HF resulted in peptide cleavage from the resin and deprotection of the side chains. Purification was executed first by Sephadex G-15 exclusion chromatography then by RP-HPLC. The purity of the peptides was revealed by analytical RP-HPLC and amino acid analysis, and FAB-MS confirmed peptide molecular weight identical with those expected (**Table 1**).

The pollen CaM (pCaM) is provided by Professor Ting Fang ZHANG (College of

Life Science, Peking University). Mw: 18,300; PI: 3.6; PDE activation: 20,000v/mg. D-pCaM was prepared according to reference 4. The concentration was 1.8 mol Dansyl/mol pCaM, determined using the method in reference 5. Fluorescence measurements were made at an excitement wavelength of 340 nm and an emission wavelength of 480 nm, on a RT-540 spectrofluorophotometer at room temperature.

Table 1. The sequences of peptides and MS/FAB analysis

Peptides	Sequences	MS/FAB (M+H)$^+$
BP-1	A – P – V – L – Q – I – K – K – T – G – S – N	1256
BP-1-(D-Ala)^{10}	A – P – V – L – Q – I – K – K – T – (D)A– S – N	1270
BP-2	(D)A – P – V – L – Q – I – K – K – T – G – S – N-NH_2	1255
BP-13	A – M – A – L – A – L – K – K – T – G -NH_2	1003
BP-14	W – A – M – A – L – A – L – K – K – T – G -NH_2	1189
BP-15	W – P – A – M – A – L – A – L – K – K – T – G -NH_2	1286

Based on the hypothesis that the binding site of CaM-antagonistic peptide is the basic amino acid cluster and an adjacent hydrophobic 6-residue segment[6], the second structure propensity (P_α, P_β) and hydrophobicity of the 6-residue segment ($\overline{K-D_6}$) adjacent to KK basic unit in these synthesized peptides were calculated in light of Chou-Fasman structure prediction[7] and method described by Kyte-Doolittle[8] (**Table 2**). The interaction between pCaM and synthesized peptides was studied making use of the particular fluorescence property of D-pCaM[9], which is found to have almost the same bioactivity as the native protein. Conformational change resulting form the binding of D-pCaM and peptides in the presence of Ca^{2+}, altered D-pCaM fluorescence spectra obviously, which did not happen when EDTA existed (**Figure 1**). The facts demonstrated that these peptides could bind pCaM and such interaction was Ca^{2+} dependent. The dissociation constant Kd values of D-pCaM-peptide complexes were calculated from data of fluorescence titration (**Figure 2**) using the following equation[10]:

$$Kd / (1-\alpha) = [peptide] / \alpha - [D\text{-}pCaM]$$

$$\alpha = (F - F_0) / (F_\infty - F_0)$$

From the Kd data presented in **Table 2**, we can see BP-13, whose α-helix propensity ($P_\alpha > P_\beta$) and hydrophobicity are higher, has the smallest Kd of 4.0 nmol/L and the highest affinity for pCaM. Although BP-14 and 15 have the same P_α, P_β and $\overline{K-D_6}$, their increased chain length may prevent them from fitting the peptide-binding pocket of pCaM so well as BP-13 did. Besides the structural factors mentioned above, we also found the configuration of amino acid residue could influence peptide affinity for pCaM greatly. When substitution of D-Ala for Gly was made at position 10 and that of D-Ala for L-Ala at position 1 in BP-1 to obtain BP-1-(D-Ala)^{10} and BP-2 respectively, the affinity for pCaM, in both cases, changed from none of BP-1 to as high as showed by Kd at less than μmol/L magnitude. This suggests an efficient access to improve peptidic antagonist's ability to associate with CaM.

We compared Kd values of peptide-D-pCaM complexes with those of peptide-D-bCaM complexes (**Table 2**), which demonstrated the two kinds of interactions were similar but differed slightly on affinity and sensitivity to structure changes. It can be attributed to the subtle difference in sequence and structure of two CaMs, manifesting the variability of CaMs from different origins although they are highly conserved.

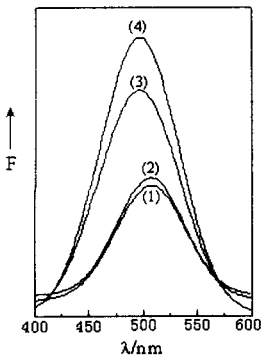
It is commonly believed that, by activating some important enzymes, CaM works in cell as the initiator of many regulatory cascade reactions[11]. We studied the effects of CaM-binding peptides on the extracellular acid rate (ECAR) of human T cells using Cytosensor Microphysiometer system[12], which tests the interaction between extra-compounds and cellular components. The changing pattern of peptide affinity for CaM was consistent with that of their effects on ECAR, indicating the role of CaM in cell

Table 2. α-Helical predictions, average hydropathy ($\overline{K-D_6}$) of peptides and dissociation constants (Kd) for the complexes of the peptides with CaM

Peptide	BP-1	BP-1-(D-Ala)[10]	BP-2	BP-13	BP-14	BP-15
P_α *	1.08	1.08	1.08	1.36	1.36	1.36
P_β *	1.18	1.18	1.18	1.02	1.02	1.02
$\overline{K-D_6}$ **	1.53	1.53	1.53	2.48	2.48	2.48
Kd (μmol/L) pCaM	—	2.9×10^{-2}	2.9×10^{-1}	4.0×10^{-3}	1.146	9.2×10^{-1}
Kd (μmol/L) bCaM	—	1.7	1.9	4.6×10^{-2}	1.3×10^{-1}	3.8×10^{-1}

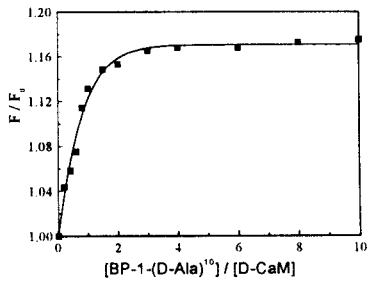
* P_α and P_β, representing respectively the formation potential of α-helix and β-sheet conformation, are the average values only for the 6 amino acid residues on the left of basic cluster (KK);
** $\overline{K-D_6}$ stands for the average hydropathic index of 6-residue segment adjacent to the basic residues (KK).

Figure 1. Fluorescence emission spectra



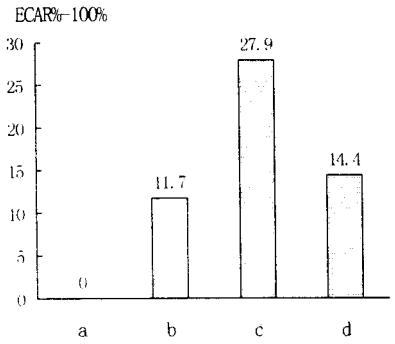
(1): 1.0μmol/L D-pCaM, 2.0mmol/L EDTA; (2): 1.0μmol/L D-pCaM, 2.0mmol/L EDTA, 1.0μmol/L BP-13; (3): 1.0μmol/L D-pCaM, 2.0mmol/L $CaCl_2$; (4): 1.0μmol/L D-pCaM, 2.0mmol/L $CaCl_2$, 1.0μmol/L BP-13. The solution also contained 0.2mmol/L KCl, 20mmol/L Hepes, pH=7.3, 25 °C.

Figure 2. Fluorescence titration curve of dansyl pollen calmodulin with peptide in the presence of Ca^{2+}.



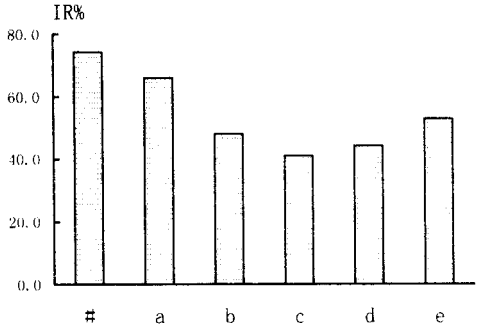
The fluorescence intensity was measured at 480nm, with 340nm excitation. The solutions also contained 1.0μmol/L D-pCaM, 2.0mmol/L $CaCl_2$, 0.2mmol/L KCl, and 20mmol/L Hepes, pH=7.3, 25 °C.

Figure 3. Effect of peptides on ECAR of T-cell measured by cytosensor microphysiometer



a. BP-1; b. BP-1-(D-Ala)[10]; c. BP-13; d. BP-14

Figure 4. K562 cell IR% measured by MTT method



Cisplatin; a. BP-1; b. BP-1-(D-Ala)[10]; c. BP-2; d. BP-13; e. BP-14

signal transduction which causes changes in cell metabolism and then in ECAR (**Figure 3**). In addition, most of the peptides showed inhibitory tendency on tumor cell K562 proliferation in MTT method[13] (**Figure 4**), suggesting their potential use in the research of antitumor medication through further improvement [14].

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