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Inhibition of calcium-calmodulin complex formation by vasorelaxant basic dipeptides demonstrated by *in vitro* and *in silico* analyses

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ABSTRACT

Background: Tryptophan-histidine (Trp-His) was found to suppress the activity of the Ca^{2+}/calmodulin (CaM)-dependent protein kinases II (CaMKII), which requires the Ca^{2+}-CaM complex for an initial activation. In this study, we attempted to clarify whether Trp-His inhibits Ca^{2+}-CaM complex formation, a CaMKII activator.
Methods: The ability of Trp-His and other peptides to inhibit Ca^{2+}-CaM complex formation was investigated by a Ca^{2+}-encapsulation fluorescence assay. The peptide-CaM interactions were illustrated by molecular dynamic simulation.
Results: We showed that Trp-His inhibited Ca^{2+}-CaM complex formation with a 1:1 binding stoichiometry of the peptide to CaM, considering that Trp-His reduced Hill coefficient of Ca^{2+}-CaM binding from 2.81 to 1.92. His-Trp also showed inhibitory activity, whereas Trp + His, 3-methyl His-Trp, and Phe-His did not show significant inhibitory activity, suggesting that the inhibitory activity was due to a peptide skeleton (irrespective of the sequence), a basic amino acid, a His residue, the N hydrogen atom of its imidazole ring, and Trp residue. *In silico* studies suggested the possibility that Trp-His and His-Trp interacted with the Ca^{2+}-binding site of CaM by forming hydrogen bonds with key Ca^{2+}-binding residues of CaM, with a binding free energy of −49.1 and −68.0 kJ/mol, respectively.
Conclusions: This is the first study demonstrating that the vasoactive dipeptide Trp-His possesses inhibitory activity against Ca^{2+}-CaM complex formation, which may elucidate how Trp-His inhibited CaMKII in a previous study.
General significance: The results provide a basic idea that could lead to the development of small peptides binding with high affinity to CaM and inhibiting Ca^{2+}-CaM complex formation in the future.
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1. Introduction

Calmodulin (CaM) is a regulatory protein involved in a variety of cellular Ca^{2+}-dependent signaling pathways. Upon binding up to four Ca^{2+} in its four EF hands, converting it to an active form as Ca^{2+}-CaM complex, CaM undergoes a conformational change, enabling it to activate a number of intracellular kinases such as Ca^{2+}/CaM-dependent protein kinase II (CaMKII) [1]. Recent reports by Prasad *et al.* [2] and Kobayashi *et al.* [3] revealed the physiological importance of CaMKII in the vasomotor response of vascular smooth muscle cells (VSMCs), in which CaMKII plays a role in the phosphorylation of voltage-dependent L-type Ca^{2+} channels (VDCCs), and inhibition of CaMKII reduces intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) elevated by angiotensin II. In a Ca^{2+}-CaM dependent manner, CaMKII undergoes initial autophosphorylation, excessive activity of which could cause chronic diseases such as atherosclerosis, hypertension, cardiac hypertrophy, and cancer [4–7]. Considering these observations, it seems likely that excessive Ca^{2+}-CaM-mediated signaling appears to be involved in those diseases. Therefore, agents that bind to CaM, inhibit Ca^{2+}-CaM complex formation, and prevent binding of the Ca^{2+}-CaM complex to CaMKII may reduce the risk of onset or progression of such diseases.

In addition to drugs, some researchers found that natural compounds, including phytochemicals and peptides, have the potential to regulate intracellular Ca^{2+}-signaling systems by inhibiting CaMKII activity and/or Ca^{2+}-CaM complex formation [3,4,8]; however, the underlying mechanism(s) and the compounds structures have not been reported. Aluko [4] reported the ability of peptides from pea protein hydrolysates to inhibit CaMKII activity competitively; however, the candidate molecules have still not been identified. In previous studies on physiological functions of small peptides [3,9–12], we have demonstrated that a vasorelaxant and anti-atherosclerotic dipeptide, tryptophan-histidine (Trp-His), inhibited the phosphorylation of VDCCs in VSMCs through lowering the CaMKII activity [3]. This finding led us to investigate whether the basic peptide is involved in the formation of a CaMKII activator, Ca^{2+}-CaM complex, because some peptides termed calcium-like peptides (CALPs, e.g., VKFGVGFKVMVF) that contain basic amino acids were reported to inhibit Ca^{2+}-CaM complex

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formation subsequent to inhibition of Ca^{2+}/CaM-dependent enzymes [13]. In this study, a Ca^{2+}-encapsulation fluorescence assay was used to investigate the ability of Trp-His and other peptides to inhibit Ca^{2+}-CaM complex formation. Having the advantage of providing useful information for the binding configuration in atomic level interaction, *in silico* analysis by molecular dynamics (MD) simulation that can predict the binding of ligands with proteins in a virtual water environment [14] was performed to evaluate the dipeptide-CaM protein complex.

2. Materials and methods

2.1. Reagents

CaM from bovine brain was obtained from Genway (San Diego, CA). Stains-all and Chelex 100 were obtained from Sigma Chemical (St. Louis, MO). Fluo-4 was purchased from Invitrogen (Eugene, OR). Trp-His, His-Trp, 3-methyl His-Trp (His[3-Me]-Trp), Phe-His, and Gly-Gly were synthesized using the 9-fluorenylmethoxycarbonyl-solid phase synthesis method according to the manufacturer's instructions (Kokusan Chemicals, Tokyo, Japan). L-Tryptophan (Trp), L-histidine (His), and 3-(*N*-morpholine)propanesulfonic acid were purchased from Nacalai Tesque (Kyoto, Japan). *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES) and ethylene glycol tetra acetic acid (EGTA) were purchased from Dojindo (Kumamoto, Japan). Working buffers and Milli-Q water were treated with a Chelex 100 column (0.9 cm × 30 cm) to remove any contaminating Ca^{2+} from all solutions. Apparatuses were thoroughly washed with 0.2 M EGTA and Chelex-treated Milli-Q water prior to experiments. Other reagents were of analytical-reagent grade and used without further purification.

2.2. Fluorescence assay for Ca^{2+}-CaM complex formation

A fluorescence assay for the formation of Ca^{2+}-CaM complexes was conducted with a Ca^{2+}-chelating fluorescence reagent, Fluo-4, by which free Ca^{2+} is specifically chelated and a characteristic fluorescence signal is produced [15]. Briefly, an aliquot (10 μL) of a given sample (Trp-His, His-Trp, Trp + His, His[3-Me]-Trp, Phe-His, Stains-all, or Gly-Gly) in 10 mM HEPES buffer (pH 7.5) was added to 10 μL of a 2.5-μM CaM solution dissolved in Chelex-treated Milli-Q water in a 96-well, half-area, flat-bottom microplate (Greiner Bio-One Ltd., Stonehouse, UK) and then incubated at 37 °C for 15 min. Thereafter, 10 μL of a 12-μM $CaCl_2$ solution was added to the mixtures. After 15 min of incubation, 10 μL of a 20-μM Fluo-4 solution (Chelex-treated Milli-Q water) was added to the mixtures. The resulting fluorescence intensity (FI) was measured using a fluorescence spectrophotometer (Wallac ARVO^{TM}SX 1420 Multilabel Counter; Perkin-Elmer Life Sciences, Tokyo, Japan) at an excitation (Ex) wavelength of 492 nm and an emission (Em) wavelength of 520 nm. Sample solution without sample was used as a control, whereas a sample solution without CaM was used as a blank. The ability of the sample to bind to CaM or to inhibit Ca^{2+}-CaM complex formation was indirectly evaluated by comparing the difference in the FI of the sample (ΔFI_{sample}: $FI_{sample\ blank} - FI_{sample}$) with that of the control ($\Delta FI_{control}$: $FI_{control\ blank} - FI_{control}$) using the following equation:

$$Ca^{2+}\text{–CaM complex}\ (\%) = \left(FI_{sample\ blank} - FI_{sample}\right)/\left(FI_{control\ blank} - FI_{control}\right) \times 100 \quad (1)$$

where $FI_{control\ blank}$, $FI_{control}$, and FI_{sample} represent the FI of Ca^{2+}-Fluo-4 products in the absence of both sample and CaM, in the absence of sample but in the presence of CaM, and in the presence of both sample and CaM, respectively. $FI_{sample\ blank}$ represents the FI in the absence of CaM and was used to account for the non-specific effect on Ca^{2+}-Fluo-4 fluorescence by the sample. Thus, if the sample (or peptide) did not inhibit Ca^{2+}-CaM complex formation, the ratio of the Ca^{2+}-CaM complex would be 100% compared with the control. Stains-all (a calcium-mimic dye that specifically binds to the Ca^{2+} binding sites of CaM [13,16]) and Gly-Gly were used as positive control and negative control, respectively. In addition, the $\Delta FI_{control}$ value obtained with 20 μM of Fluo-4 (Ca^{2+}-CaM complex in the absence of sample but in the presence of 2.5 μM CaM) increased with increasing Ca^{2+} concentration (4 to 12 μM), indicating that under the present assay conditions, 12 μM Ca^{2+} with a maximal $\Delta FI_{control}$ value of 5.4×10^4 was useful in evaluating the inhibitory activity of the samples on Ca^{2+}-CaM complex formation (data not shown).

2.3. Hill plot analysis

Hill plots of the binding of Ca^{2+} to CaM were constructed to evaluate the interaction of a dipeptide, Trp-His, with CaM; the Hill coefficient (n_{Hill}) was calculated from the Hill plot using the following equation [17]:

$$\log(Y/(1-Y)) = n\log\left[Ca^{2+}\right]_{free} - n\log K \quad (2)$$

where n is the Hill coefficient, K is the macroscopic dissociation constant, which represents the sum of the microscopic equilibrium binding constants for homotropic cooperativity [17], Y is the fractional saturation of CaM, and $[Ca^{2+}]_{free}$ is the CaM-unbound Ca^{2+} concentration. To construct the Hill plot using Eq. (2), $[Ca^{2+}]_{free}$ and Y were obtained from Eqs. (3) and (4) as follows:

$$\left[Ca^{2+}\right]_{free} = \left(\left[Ca^{2+}\right]_{total} \text{x } FI_{sample}\right)/FI_{sample\ blank} \quad (3)$$

where $[Ca^{2+}]_{total}$ is the total amount of Ca^{2+} added. Y was calculated as follows:

$$Y = \left[Ca^{2+}\right]_{bound}/4[CaM] \quad (4)$$

where $[Ca^{2+}]_{bound}$ is the concentration of CaM-bound Ca^{2+}, which was calculated as $[Ca^{2+}]_{bound} = [Ca^{2+}]_{total} - [Ca^{2+}]_{free}$, 4 is the total number of Ca^{2+}-binding sites of CaM [1], and [CaM] is the CaM concentration. Thus, Y and $[Ca^{2+}]_{free}$ were fitted to Eq. (2) to construct the Hill plots and calculate n_{Hill}. For the assay, 10 μL of 600 μM Trp-His (150 μM, final concentration) in 10 mM HEPES buffer (pH 7.5) was added to 10 μL of a 2.5-μM CaM solution (CaM dissolved in Chelex-treated Milli-Q water), followed by incubation at 37 °C for 15 min. Then, 10 μL of a 40-μM $CaCl_2$ solution in 10 mM HEPES buffer (pH 7.5) was added to the mixture. After 15 min of incubation, 10 μL of 20 μM Fluo-4 in Chelex-treated Milli-Q water was added to the samples, followed by FI measurements at 492 nm (Ex) and 520 nm (Em). Data were fitted with GraphPad Prism version 5 software (La Jolla, CA).

2.4. MD simulation analysis of dipeptide-CaM complex

Initial structures of dipeptides (Trp-His and His-Trp) were built using Chem3D ver.7.0, and their stable conformations in water were calculated on the basis of the AM1 closed-shell theory in MOPAC [14]. Considering that, around pH 7, an imidazole ring on His is partially protonated [18], a protonated form of His was used in this simulation. Ca^{2+}-loaded CaM (PDB code 1PRW) was used as a template for docking experiments with dipeptides. Before performing the docking experiments, the initial protein structure of CaM was modified by the SYBYL X2.0/Biopolymer tool as follows: water molecules and Ca^{2+} atoms were removed from the initial structure and the amino acids in Ca^{2+}-binding loop I of CaM (Asp^{20}, Lys^{21}, Asp^{22}, Gly^{23}, Asp^{24}, Gly^{25}, Thr^{26}, Ile^{27}, Thr^{28}, Thr^{29}, Lys^{30}, and Glu^{31}) were optimized to be in their ionized forms at neutral pH. Docking of experiments of the peptides with the Ca^{2+}-binding loop I were performed with the SYBYL/Surflex-dock program. MD simulations of peptide-CaM complexes were performed

with the AMBER 12 package program to calculate the binding energies and changes in interatomic distances. These complexes were surrounded by TIP3P water molecules forming a truncated octahedron box in which the protein atom was at least 12 Å away from the nearest edge of the box. These complex systems were minimized prior to running MD simulations in the following steps. First, the positions of water and counterions were optimized in the AMBER force field 99SB with the protein being fixed. Second, entire systems were minimized and heated up to 300 K for 100 ps. Then, MD simulations of these systems were performed for 2500 ps by 2 fs time steps with constant temperature and pressure (300 K and 1 atm). Molecular cartoons were drawn with a visual MD software to visualize the binding of the peptides to CaM in water. Binding free energies (ΔG_{bind}) of peptides with CaM were calculated with the molecular mechanics Poisson-Boltzmann surface area method [14] as a sum of van der Waals, electrostatic interaction, and solvation free energies on the extracted snapshots during 2000 ps and 2500 ps of MD trajectories.

2.5. Statistical analysis

Results are expressed as mean ± SEM values. Statistical differences between two groups were analyzed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant. All analyses were performed with the StatView J 5.0 software (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Inhibition of Ca^{2+}-CaM complex formation by Trp-His

In a series of studies on dipeptide-induced vasorelaxant and anti-atherosclerotic effects, possible underlying mechanisms have been revealed, by which Trp-His exerted physiological effects through the suppression of CaMKII/phosphorylation of VDCCs in VSMCs [3]. This finding led us to investigate whether the peptide interferes with the Ca^{2+}-CaM complex formation step, because CaMKII activation is initiated by the Ca^{2+}-CaM complex [4]. Fig. 1 shows that the ratio of the Ca^{2+}-CaM complex was significantly ($P < 0.01$) and dose-dependently decreased by Trp-His (150–500 μM) as well as Stains-all, which preferably binds to Ca^{2+}-binding sites of CaM [13,16]. This result suggests that Trp-His inhibits the formation of Ca^{2+}-CaM complexes.

Hill plot analysis provided useful information about the Trp-His effect on Ca^{2+}-CaM complex formation (Fig. 2).

In a control experiment (without Trp-His), the binding of Ca^{2+} to CaM exhibited an n_{Hill} of 2.81 ($R^2 = 0.998$) with a K_d of 11.5 μM, providing evidence for positive cooperativity in the binding of Ca^{2+} to CaM and implying that Ca^{2+} binds to three of the four Ca^{2+}-binding sites of CaM. The results fall within the range of values reported previously, where the Ca^{2+}:CaM stoichiometry was in the range from 1.33 to 4.1 [17–22] and K_d ranged from 3 to 20 μM for the binding of Ca^{2+} to CaM [21]. In the presence of Trp-His, n_{Hill} was reduced to 1.92 ($R^2 = 0.919$) accompanied by an increase in K_d (17.0 μM), demonstrating that the cooperative interaction of Ca^{2+} with CaM was still present but diminished in the presence of Trp-His. The reduction of n_{Hill} from 2.81 to 1.92 in the presence of Trp-His implied that the number of Ca^{2+} ions that bound to CaM was reduced from three to two. Taken together, Trp-His may be a potent natural compound with activity against an upstream CaMKII activator, i.e., Ca^{2+}-CaM complex, unlike other compounds that directly inhibit CaMKII, e.g., trideca-peptide [23] and phytochemicals [8,24].

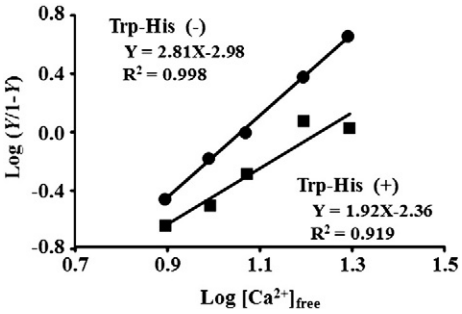

Fig. 2. Hill plots of Ca^{2+} binding to calmodulin in the absence (●) and presence (■) of Trp-His Changes in fluorescence intensities of 5 μM Fluo-4 at 492 nm (Ex) and 520 nm (Em) were determined to assess the binding of Ca^{2+} to calmodulin (CaM) in the absence and presence of 150 μM Trp-His. Y corresponds to the fractional saturation of CaM with Ca^{2+}.

3.2. Structure-activity relationship

In order to determine the structural requirements of Trp-His to inhibit Ca^{2+}-CaM complex formation, His-Trp, His[3-Me]-Trp, Phe-His, and their corresponding amino acid mixture (Trp + His) were used in this experiment. As shown in Fig. 1, His-Trp was as potent in inhibiting Ca^{2+}-CaM complex formation (30.7 ± 4.9% reduction) as Trp-His (30.8 ± 2.0% reduction). In contrast, the mixture of their corresponding amino acids, His[3-Me]-Trp, and Phe-His did not show any significant reduction in the ratio of Ca^{2+}-CaM complex, similar to Gly-Gly. These findings strongly indicate that (1) both basic dipeptides may prevent binding of Ca^{2+} to CaM, irrespective of their sequences; and (2) a peptide skeleton, His residue or the N hydrogen atom of its imidazole ring, and Trp residue are essential to exert the effect.

To date, some binding characteristics of compounds that bind to CaM have been reported. Villain *et al.* [13] reported that CALPs directly competed with Ca^{2+} to bind to Ca^{2+}-binding sites and thereby inhibited Ca^{2+}-CaM complex formation, leading to the suppression of phosphodiesterase activity. Sasaki *et al.* [19] demonstrated that ruthenium red bound to CaM at sites different from the Ca^{2+}-binding sites, having an allosteric inhibitory effect on the interaction of Ca^{2+} with

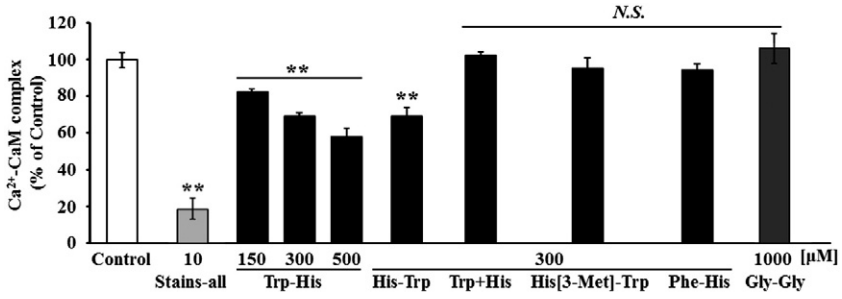

Fig. 1. Inhibitory effect of dipeptides on Ca^{2+}-calmodulin complex formation. The inhibitory effect of the samples against the formation of the Ca^{2+}-calmodulin (CaM) complex was evaluated by a measurement of free Ca^{2+} specifically chelated by 5 μM Fluo-4 at 492 nm (Ex) and 520 nm (Em). The concentration of each dipeptide was set at 300 μM, except of Trp-His (150–500 μM), Stains-all (10 μM), and Gly-Gly (1000 μM). CaM and $CaCl_2$ concentrations were 0.63 μM and 3 μM, respectively. Values are mean ± SEM (**$p < 0.01$ vs. control, $n = 3$; N.S., not significant).

CaM subsequent to inhibition of Ca^{2+}/CaM-dependent enzymes and smooth muscle contraction. Thus, the next aim of this study was to identify the mechanism by which active dipeptides (Trp-His and His-Trp) bind to CaM by MD simulation.

3.3. MD simulation analysis of dipeptide-CaM complexes

Because acidic amino acids are abundant in Ca^{2+}-binding sites of CaM [16,25] and the *in vitro* inhibitory effect of the basic dipeptides Trp-His and His-Trp on Ca^{2+}-CaM complex formation was demonstrated (Figs. 1 and 2), we hypothesized that these dipeptides may bind with high potency to the Ca^{2+}-binding sites of CaM. In order to clarify whether the peptides are able to interact with Ca^{2+}-binding sites, MD simulation analysis was performed.

As a result, MD simulation of the docking complex (Fig. 3A) illustrated that Trp-His interacted with the Ca^{2+}-binding site (loop I) of CaM during 2500 ps, suggesting that Trp-His preferably bound to the Ca^{2+}-binding site. In addition, the backbone root-mean-square deviation (backbone RMSD) from the start structure was ≤3.0 Å (Fig. 3G), indicating good structure stability and successful simulation. The zoomed view snapshot shown in Fig. 3C displays the binding configuration, in which the peptide-CaM complex was stabilized by hydrogen bonds (blue lines in Fig. 3C and E) between a carboxyl group ($-COO^-$) of Asp^{22} of CaM, an amino group ($-NH_3^+$) of Trp (2.7–2.9 Å), and an imidazole protonated-δ1-nitrogen ($-N^{\delta1}-H^+$) of His (2.7–2.8 Å) of the peptide. Similarly, MD simulation showed the interaction of His-Trp with CaM (Fig. 3B) with a backbone RMSD of ≤2.5 Å (Fig. 3G). The complex was stabilized by hydrogen bonds (blue lines in Figs. 3D and F) between $-COO^-$ of Asp^{22} of CaM and $-N^{\delta1}-H^+$ (3.0–6.0 Å) and $-NH_3^+$ (2.7–3.0 Å) of the His residue of the peptide and between $-COO^-$ of Glu^{31} of CaM and $-NH_3^+$ (2.7–3.0 Å) of the His residue of the peptide. These hydrogen bonding distances are consistent with the reported hydrogen bonding distance for NH · · · O of 2.89 Å [26]. In addition, these MD simulations revealed the importance of the His residue and the N hydrogen atom of the imidazole ring of His for inhibiting Ca^{2+}-CaM complex formation, as predicted in Fig. 1. The interaction between CaM-dipeptides by MD simulation may also be supported by reports that showed that the positively charged group of compounds and the nitrogen atom in the aromatic ring plays a key role in interacting with CaM by facilitating electrostatic interactions and forming hydrogen bonds [27,28]. Although the role of Trp in the interaction was not observed by MD simulation, investigations on the importance of the Trp residue and other essential features of peptides that inhibit Ca^{2+}-CaM complex formation as well as screening of potent peptides are currently in progress.

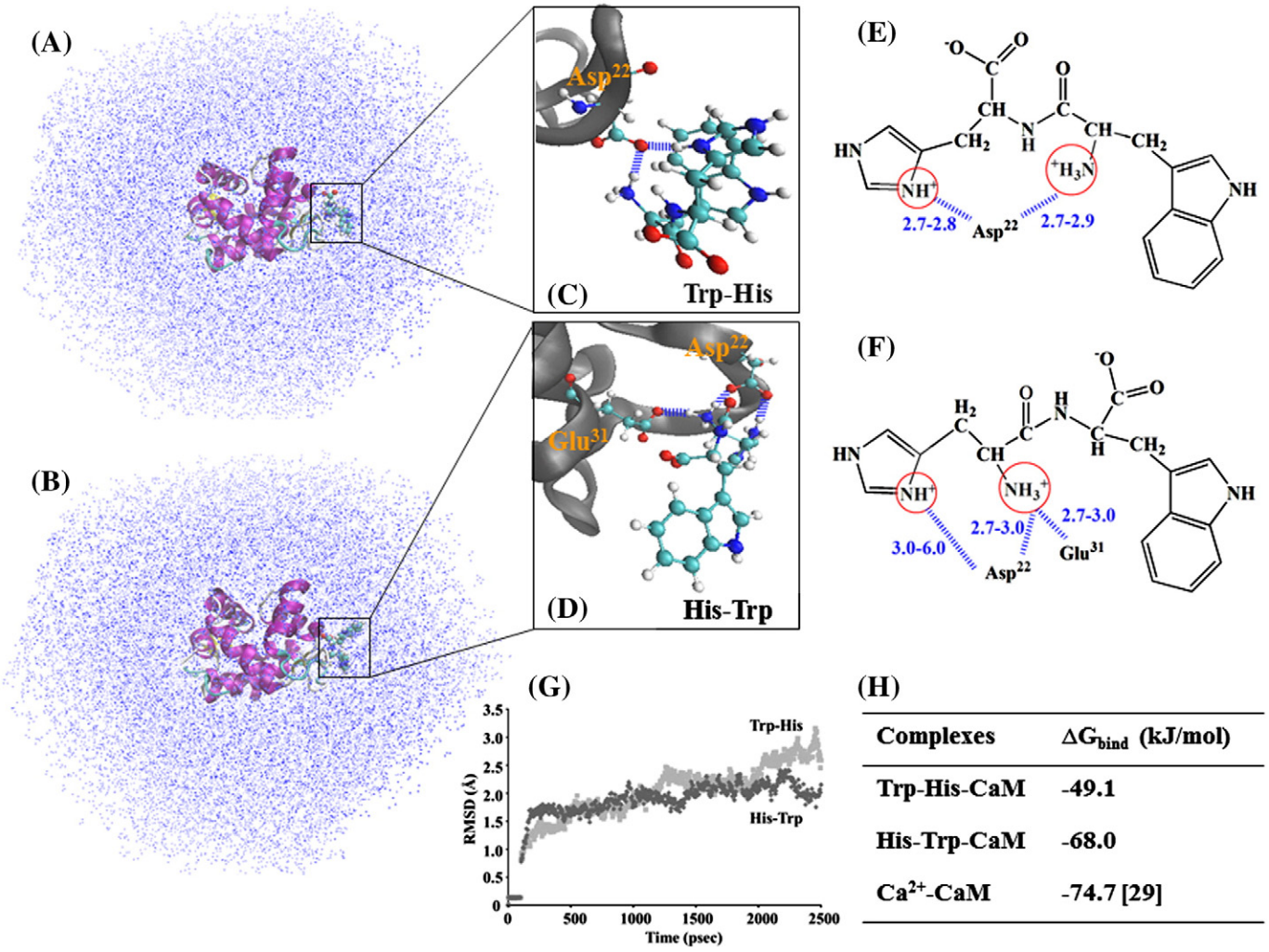

Fig. 3. MD simulation analyses of basic dipeptides with Ca^{2+}-binding loop I in calmodulin Virtual structures of the Trp-His-calmodulin (CaM) complex (A) and His-Trp-CaM complex (B) surrounded by water molecules, in which the purple ribbon model and the stick model represent CaM and peptides, respectively. Inset, the key amino acid residues of CaM (labeled orange in the stick model) forming hydrogen bonds (blue lines) with Trp-His (C) or His-Trp (D). The corresponding colors are as follows: C atom, cyan; H atom, white; N atom, blue; and O atom, red. Hydrogen bonds forming moieties of Trp-His (E) or His-Trp (F) with CaM and their binding distances (Å). A backbone root-mean-square deviation (backbone RMSD) from the initial structure is shown in (G). Binding free energies, ΔG_{bind} (kJ/mol), of the Trp-His-CaM complex, His-Trp-CaM complex, and Ca^{2+}-CaM complex are summarized in (H).

As shown in Fig. 3H, the theoretical estimation of ΔG_{bind} of the Trp-His-CaM complex was −49.1 kJ/mol (van der Waals force: −16.4 kJ/mol, electrostatic interaction energy: −1764.0 kJ/mol, and solvation free energy: 1731.3 kJ/mol), indicating stable binding of the complex. Likewise, ΔG_{bind} of the His-Trp-CaM complex was similar (−68.0 kJ/mol) to that of the Trp-His-CaM complex. Our estimate for the ΔG_{bind} value is less negative than that reported by Linse *et al.* (−74.7 kJ/mol) [29] for the binding of Ca^{2+} to Ca^{2+}-binding sites in the N-terminal domain of CaM, suggesting that Trp-His and His-Trp preferably bind to CaM, but the binding affinity was weaker than that of Ca^{2+} to CaM. The ΔG_{bind} value for the peptide-CaM interactions should give approximate K_d in nanomolar levels, which is different from the concentration levels (micromolar levels) exhibiting the inhibitory effect in the fluorescence assay. The difference of the dipeptide affinity levels obtained from both experiments would be due to the difference of the experimental conditions, in which MD simulations were performed in the absence of Ca^{2+}, while the fluorescence assays were done in the presence of Ca^{2+}. In addition, the low dipeptide affinity observed from the fluorescence assay may be due to a small fraction of the protonated form of Trp-His at the experimental pH 7.5. In the fluorescence assay at pH 7.5, the imidazole ring of His on Trp-His was predominantly in the deprotonated form. By the MD simulation analysis, the deprotonated form was predicted to have less negative ΔG_{bind} of −32.0 kJ/mol (approximate K_d in micromolar levels) than that of the protonated form (−49.1 kJ/mol, approximate K_d in nanomolar levels), suggesting the weaker binding affinity of the deprotonated Trp-His to CaM; in turn, the deprotonated Trp-His has about three orders of magnitude lower affinity than that of the protonated Trp-His.

Together with the results of the fluorescence assay and Hill plot analysis, these results suggest that Trp-His might specifically bind to the Ca^{2+}-binding site of CaM. This mechanism of CaM inhibition by Trp-His or His-Trp may be different from that reported previously for other CaM antagonists inhibiting CaM by binding to hydrophobic pockets of CaM, but not to Ca^{2+}-binding sites [30]. These findings may clarify the mechanism underlying the action of Trp-His on the suppression of Ca^{2+}-CaM/CaMKII/VDCC phosphorylation pathway in VSMCs from the previous study [3], by which Trp-His prevented the binding of Ca^{2+} to CaM and hence inhibited CaM to activate CaMKII, leading to the suppression of VDCC phosphorylation. Although some scientists [31] demonstrated that just two Ca^{2+} ions binding to CaM could activate CaMKII, we suggested that a reduction in the binding number of Ca^{2+} ions to CaM from three to two by Trp-His would cause a decrease in the affinity of CaM to the enzyme, which is in good agreement with some studies [21,32,33] reporting that the binding of Ca^{2+} by three or four ions was necessary for CaM to activate Ca^{2+}/CaM-dependent enzymes. However, the inhibitory effect of the Trp-His-CaM complex on CaMK II activation should be clarified in further *in vitro* experiments. Because a mild inhibitory effect of Trp-His on Ca^{2+}-CaM complex formation was observed in this study, it appears that, in previous studies, Trp-His might exert physiological effects not only by inhibiting Ca^{2+}-CaM complex formation but also by being involved in other mechanisms such as binding to VDCCs, leading to the reduction of elevated $[Ca^{2+}]_i$ in VSMCs [11].

In conclusion, we demonstrated for the first time that the basic dipeptides Trp-His and His-Trp are a new type of CaM inhibitor that prevents CaM from forming a complex with Ca^{2+}. The inhibitory activity of the dipeptides against Ca^{2+}-CaM complex formation would be due to the basic amino acid, a His residue, the N hydrogen atom of the imidazole ring, a Trp residue, and the peptide skeleton (irrespective of the peptide sequence). In addition, the *in silico* study implied that Trp-His and His-Trp had the potency to bind to Ca^{2+}-binding sites of CaM by forming stable hydrogen bonds with the key Ca^{2+}-binding residues of Ca^{2+}-binding sites, i.e., Asp^{22} and Glu^{31}, directly preventing the binding of Ca^{2+} to CaM, with ΔG_{bind} of −49.1 kJ/mol and −68.0 kJ/mol, respectively. This preliminary study has set the stage for the development of Trp-His or His-Trp variants or other dipeptides that may be useful in the future.

Acknowledgments

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