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^{1}H NMR and Spin-Labeled EPR Studies on the Interaction of Calmodulin with Jujuboside A

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Jujuboside A (JuA), an effective component of sanzaoren, a Chinese herbal medicine, is a noncompetitive inhibitor of calmodulin (CaM). The interaction of JuA with CaM has been investigated with ^{1}H NMR and spin-labeled EPR spectroscopies. The ^{1}H NMR experiments showed that JuA has two kinds of binding sites on CaM: one locates in the N-terminus, the other locates in the C-terminal region of the polypeptide chain. The EPR studies on 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl labeled CaM (spin-labeled CaM) revealed that each CaM molecule can bind at least two JuA molecules. Binding of JuA affects the enviroments of some lysine residues (most likely Lys-74 and Lys-94), suggesting that JuA binds to CaM through hydrophobic interaction.

Calmodulin (CaM) is a multifunctional Ca^{2+} binding regulatory protein, which exists ubiquitously in various eukaryotic cells (1). Each CaM has four Ca^{2+} binding sites, two with high affinity on the carboxyl half (sites III and IV) and the other two with low affinity at the amino half of the polypeptide chain (sites I and II). It has been found that CaM can regulate a variety of proteins and enzymes, including cyclic nucleotide phosphodiesterase, protein kinase, Ca^{2+}-Mg^{2+}-ATPase, and proteins involved in muscle contraction and hormone actions (1-3). Most of the regulatory activities of CaM are calcium-dependent, requiring binding of from one to four Ca^{2+}. The CaM-dependent activities of these enzymes and proteins can be inhibited by a diverse spectrum of inhibitors, including small peptides and many drugs, such as the

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Abbreviations: CaM, calmodulin; cAMP, cyclic adenosine monophosphate; D-CaM, dansyl-calmodulin; DPPH, 2,2-di(4-tert-octylphenyl)-1-picrylhydrazl; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; EPR, electron paramegnetic resonance; JuA, jujuboside A; NMR, nuclear magnetic resonance; PDE, phosphodiesterase; SDS, sodium dodecylsulfate; TMS, tetramethylsilane.

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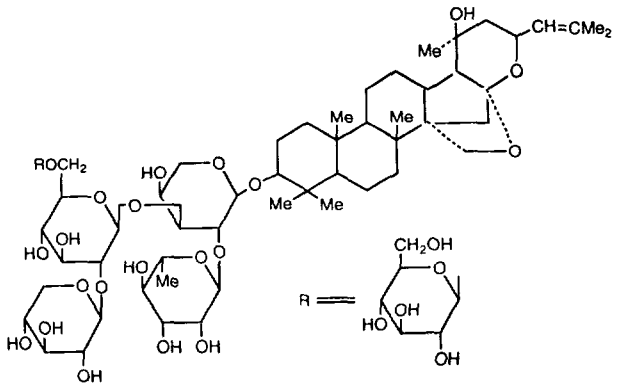


Figure 1. The structure of Jujuboside A.

phenothiazine antipsychotics (4). The modification of the activity of CaM by drugs might have profound pharmacological implications, because the study of the interaction between CaM and drug molecules may help to understand the molecular basis of these drugs and to develop new drugs. Because some of these inhibitors competitively inhibit the regulation of target enzymes by CaM, these inhibitor also serve as model compounds for the study of target protein interactions with CaM (5,6).

Cyclic nucleotide phosphodiesterase (PDE) is an important enzyme in the metabolism of second messenger cAMP. The early finding that PDE was inactivated after the purification and could be activated again by a protein led to the discovery of CaM (7). It was later known that CaM activates PDE in a calcium-dependent way. Recently, it was found that the activation of PDE by CaM was inhibited by Jujuboside A (JuA), an effective component isolated from a Chinese herbal drug called "sanzaoren" (8). This is interesting because sanzaoren, the seed of *Zizyphus jujuba*, has traditionally been used in Chinese medicine as a drug to counteract insomnia, neurasthenia and sometimes sleepiness caused by physical emaciation (9). Jujuboside A (Fig. 1) noncompetitively inhibits Ca^{2+}-dependent activation of PDE by CaM (8). The interaction of JuA with CaM has been studied using fluorescence spectroscopy of dansyl CaM(D-CaM), and the dissociation constant was determined as about 2.8 μM (10).

Because JuA has different inhibition mechanism and structural characteristics from phenothiazine and related drugs, interaction of CaM with JuA was further investigated by NMR and EPR spectroscopies. In this paper, we report the results which showed that JuA can bind to both the N- and the C-terminal regions of CaM, probably through hydrophobic interaction. In EPR studies, CaM was labeled with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl.

Experimental Procedures

Reagents. Jujuboside A is a standard product of Chinese Medicine and Biological Product Examination Institute (Beijing, PRC). 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was purchased from Sigma (St. Louis, USA).

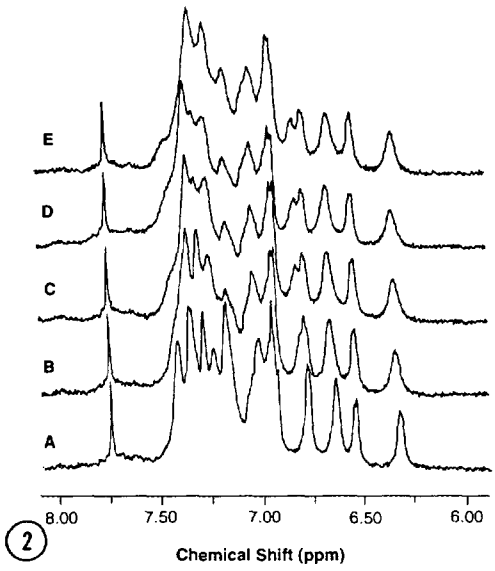
Preparation of CaM and Spin-Labeled CaM. Homogeneous CaM was purified from bovine brain by the fluphenazine-Sepharose 4B affinity chromatography(11). The purity of the protein was examined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and ultraviolet absorption spectroscopy (12). The activity of CaM was assayed by its ability to stimulate PDE in hydrolyzing cyclic 3', 5'-AMP (13). Spin-labeled CaM was prepared as reported (14). The average number of spin-labeled residues per CaM molecule was determined to be 2.2 from the secondary intergral strength of the signals of spin-labeled CaM with 2,2-di(4-tert-octylphenyl)-1-picrylhydrazl (DPPH) as standard. The spin-labeled CaM was able to activate PDE to the same level as native CaM.

NMR Spectroscopy. The purified protein was lyophilized in D_2O at least three times to replace exchangeable protons with deuteriums. For NMR measurements, all samples were dissolved in D_2O (containing 200 mM KCl and 5 mM $CaCl_2$) to a concentration of 1.0 mM. The pH value was adjusted to 7.5 (direct meter reading) by using DCl or NaOD. ^1H NMR spectra were taken at 20 °C on a Bruker AM-500 NMR spectrometer operating at 500 MHz. 5000 to 10000 transients were collected in a 5000 Hz bandwidth. The residual HDO peak was suppressed using the gated decoupling method supplied with the spectrometer and chemical shifts were measured in part per million (ppm) with TMS as an internal standard.

EPR Studies. EPR spectra of spin-labeled CaM were measured on a Bruker-EP-SRC-200 X-band spectrometer with a glass capillary tube of 1.0 mm i.d. at 22°C. The instrumental parameters are as follows: frequency 9.68 GHz, power of microwave, 5 mW; modulation frequency, 100 KHz; modulation amplitude, 1.25 Gauss.

Results

^1H NMR Studies. JuA, whose structure is shown in figure 1, consists of a hydrophobic sapogenin head and a hydrophilic glycosyl tail. The ^1H NMR spectrum of JuA shows signals from 1 to 6 ppm, with nothing in the aromatic region (6 to 8 ppm). This makes it possible to study the interaction of CaM with JuA by observing the changes in the aromatic region of the CaM ^1H NMR spectrum. The resonance signals in the downfield region (6.0 to 8.0 ppm) of the ^1H NMR spectrum of CaM can all be assigned to the one His, two Tyr, and eight Phe residues contained in this protein. The resonances of the C_δ and C_ε protons of His-107, Tyr-99 and Tyr 138, and the resonances of the aromatic protons of some of the Phe residues have been assigned (15-17). Figure 2 shows the ^1H NMR spectra of CaM titrated with JuA in the presence of Ca^{2+}. It was found that the addition of the first JuA molecule to CaM did not cause apparent changes in the chemical shifts and intensities of the resonances from His-107 and Tyr-138, except that those peaks became broader (Fig. 2B). But there were major changes in the resonance signals of the Phe residues. For example, the 7.41 ppm peak disappeared, the 6.65 ppm peak was shifted downfield, and the intensity of the 7.21 ppm peak decreased dramatically. The 6.78 and 7.29 ppm signals which were assigned to the C_δ and C_ε protons of Tyr-99 were shifted slightly downfield. When increasing the molar ratio of JuA to CaM (2:1), the signals from His-107 and Tyr-138 remained unchanged; however, the 6.78 ppm peak of Tyr-99 was split into a doublet, the intensity of the 7.29 ppm signal significantly decreased, and a shoulder peak at 7.46 ppm appeared (Fig. 2C). There were no obvious changes in the resonances of the Phe protons except broading, when going from a 1:1 to a 2:1 JuA/CaM ratio. The above changes were more pronounced with the addition of the third JuA molecule (Fig. 2D), while there were almost no changes in the ^1H NMR spectra when more than 3 equivalences of JuA were added to CaM (Fig. 1E).



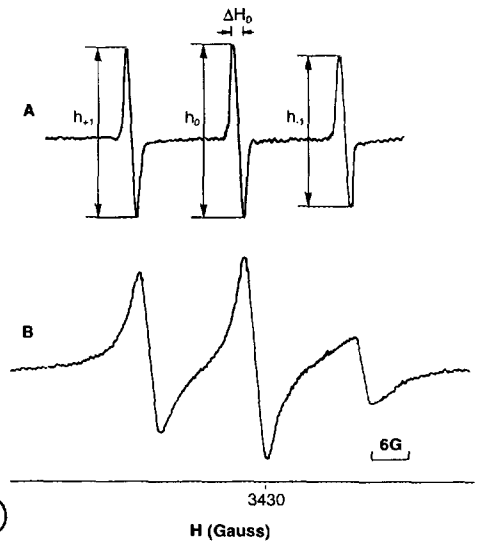


Figure 2. The effects of different JuA concentration on the ^1H NMR spectrum of CaM in D_2O containing 0.2 mM KCl and 5.0 mM $CaCl_2$ at PD 7.5. JuA:CaM mole ratio is A, 0:1; B, 1:1; C, 2:1; D, 3:1; E, 5:1.

Figure 3. EPR spectra of the spin label and spin-labeled CaM. A, Spin label in 50 mM NH_4HCO_3, pH 7.5,buffer; B, 0.1 mM spin-labeled CaM in 50 mM NH_4HCO_3,pH 7.5,buffer in the presence of 5.0 mM Ca^{2+}.

The Effect of Jujuboside A on the EPR Spectra of Spin-Labeled Calmodulin The typical EPR spectrum of an aqueous solution of free spin label contains three sharp differential absorption lines with almost equal amplitude (Fig. 3A). When the spin labels are attached to CaM, these peaks are broaden and no longer have the same intensity (Fig. 3B). This is especially evident with the highest-field peak which is broadened because of restriction of free molecular rotation. The relative rotation time (τ_R) of spin-labeled CaM was determined from the central line width and the amplitude of the three lines using the following equation (18):

$$\tau_R = 0.6\Delta H_0[(h_0/h_{+1})^{1/2}+(h_0/h_{-1})^{1/2}-2] \text{ (ns)} \qquad (1)$$

where ΔH_0 is the central line width in gauss and h_{+1}, h_0 and h_{-1} represent the relative amplitudes of the low-, middle- and high-field peaks, respectively. The value of τ_R for spin-labeled CaM is 0.97 ns when no Ca^{2+} is added (in the presence of EGTA), and 1.45 ns in the presence of Ca^{2+}. When spin-labeled CaM was titrated with JuA in the presence of Ca^{2+}, the intensity of the three peaks decreased proportionally, but the value of ΔH_0 was unchanged. Calculations of the rotational correlation time, τ_R, from equation (1) showed that the addition of up to three molecules of JuA per CaM apparently changed τ_R , but no further change was observed with more JuA (Fig. 4). However, this change of τ_R upon addition of JuA was not observed in the presence of EGTA (data not shown), indicating that the interaction of JuA with CaM is Ca^{2+}-dependent.

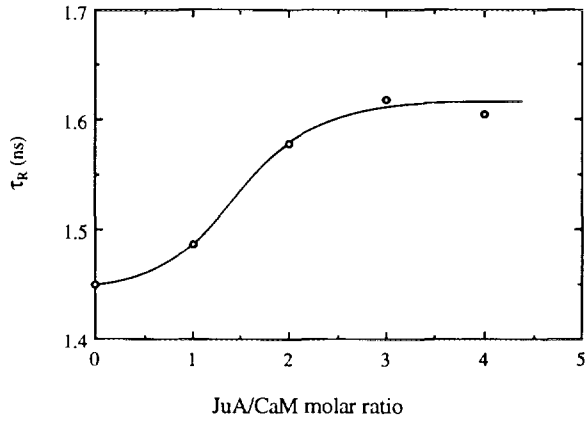
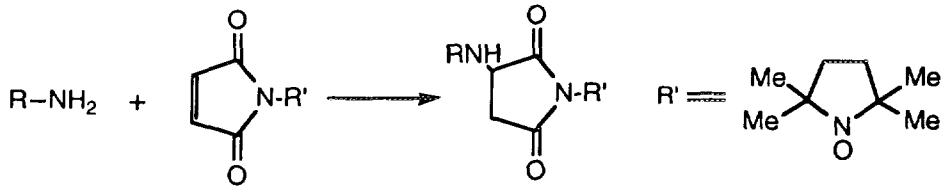


Figure 4. The effect of JuA on the rotational correlation time (τ_r) of spin-labeled CaM. 0.1 mM spin-labeled CaM in 50 mM NH_4HCO_3,pH 7.5,buffer containing 5.0 mM Ca^{2+}.

Discussion and Conclusions

The ^1H NMR titration results showed that upon addition of the first equivalence of JuA, there is no apparent changes in the NMR resonances of the ring protons of the Tyr-99, His-107, and Tyr-138 residues located in the C-terminal region of the CaM poplypeptide chain (Fig. 2). However, there are significant changes in the NMR pattern of the aromatic protons of the Phe residues, most of which (five out of eight) are located in the N-terminal part. There is strong evidence that the first JuA molecule binds to somewhere in the N-terminal part of the CaM molecule. The addition of the second JuA molecule to CaM causes no obvious change ir. the ^1H NMR resonances of the Phe residues, indicating that the second JuA molecule binds to CaM in a different manner. Since the NMR resonances of the Tyr-99 protons were altered, it may be infered that the second JuA molecule binds to somewhere close to the Tyr-99 residue. As Tyr-99 is located near the third Ca^{2+} binding site in the C-terminal region of the CaM polypeptide chain, the second JuA molecule propably binds to a place that is close to the third Ca^{2+} binding site. The similar effects on the ^1H NMR spectrum of CaM upon binding the third JuA molecule suggests that the third JuA molecule also binds to the C-terminal part of the CaM polypeptide chain. As adding more JuA does not cause further changes in the ^1H NMR spectrum of CaM, the JuA binding sites on CaM might be saturated by three JuA molecules. Based on the above results, we propose that there are two types of JuA binding sites on CaM: a high-affinity binding site located in the N-terminal region of the protein which can accommodate only one JuA molecule; and the low-affinity binding site(s) in the C-terminal region near the third Ca^{2+} binding site which can host more than one JuA molecule.

Nitroxide maleimide spin labels react mainly by nucleophilic addition with the thiol and primary amine groups in protein peptide chains. Since there are no cysteine residues in CaM, the spin labels react with lysine residues in this protein:



Therefore, the results from the labeled CaM represent conformational changes around lysine residues during interaction with JuA.

Bovine brain CaM contains 8 lysine residues, one of which, Lys-115, is trimethylated at ε-NH_2; the other seven residues are at 13, 21, 30, 75, 77, 94 and 148. It has been shown that in the presence of Ca^{2+} the most reactive primary amine groups in CaM are the ε-NH_2 of Lys-75 and Lys-94 (19,20). Because the average number of labeling residue per CaM in our experiments is 2.2 (see Experimantal Procedures), we believe that CaM was labeled mainly at Lys-75 and Lys-94.

The EPR results showed that the increases of τ_R induced by the addition of the first two JuA per CaM acccount for 82% of the maximal change observed (Fig. 3), supporting the earlier evidence that more than one molecules of JuA can bind to a CaM molecule. JuA probably binds through its sapogenin head to the hydrophoic areas at both ends of CaM. It is worth noting that the change of τ_R induced by the addition of the second JuA was the most dramatic, thus implying that the binding site of the second JuA is very close to a spin-labeled lysine residue. From our ^1H NMR studies, it is clear that the first JuA binds to the hydrophobic area at N-terminal region, thereby affecting the side chain of Lys-75. Therfore, the residue affected by the binding of second JuA is likely the C-terminal Lys-94 (as discussed above). From these results, we conclude that JuA has at least two binding sites on CaM, one at the N-terminal region with high affinity, and another at the C-terminal region with lower affinity.

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