Abstract—The effects of the Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Mn$^{2+}$ on the conformation of calmodulin (CaM) have been tested by using 400 MHz $^1$H NMR. In the aromatic region of the spectrum of CaM with a one molar ratio of cation per CaM, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Mn$^{2+}$ induced spectral changes which were very similar to those seen for Ca$^{2+}$. However, the Mg$^{2+}$-induced change was different. These results are consistent with our pharmacological findings on divalent cations.

It is generally accepted that the mechanism of action by which the calcium modulator protein calmodulin (CaM) regulates Ca$^{2+}$-dependent functions involves a conformational change in the protein which occurs with Ca$^{2+}$-binding. The binding of Ca$^{2+}$ ions, as well as several other divalent cations, i.e., Mg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$ to CaM has been demonstrated by both direct and indirect measurements [(1), for a review; (2, 3)]. Biogenic amines such as serotonin (5-HT), dopamine (DA) and norepinephrine (NE) are known to control many neuron functions. Recently, it has been demonstrated by an in vitro test that the biogenic amine synthesizing enzymes were regulated by calcium ions via calcium-CaM-dependent protein kinase (4, 5). Also, we have suggested by a behavioral test in mice that Ca$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$ activated the enzyme that synthesizes 5-HT, DA and NE via cerebral CaM; however, Mg$^{2+}$ did not affect this enzyme (6-9). Thus divalent cation induced conformational change of CaM has been tested by using 400 MHz $^1$H NMR to confirm our results of this behavioral test. It would be unexpected to confirm such an animal experiment at the molecular level by NMR. However, we report in this communication our results of $^1$H NMR spectroscopy of CaM with Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Mn$^{2+}$ which are consistent with the finding of our behavioral test.

CaM was prepared from bovine brain by a modification of the procedure by Yazawa et al. (10). The final purification of the protein was performed by chromatography on a 0.3×3 cm Bio Gel P-2 column equilibrated with doubly distilled deionized water. The protein was judged to be at least 95% pure from its pattern on 7.5% polyacrylamide gels electrophoresed with a tris-glycine continuous buffer system (11).

The cation-free protein was prepared by dialyzing 5.0 ml CaM (4 mg/ml) for 24 hr against one liter of 1 mM EDTA, pH 7.3. This was followed by dialyzing the protein against two changes of doubly distilled deionized water. The free Ca$^{2+}$ level of the doubly distilled deionized water was less than 0.7 μM. The calcium content was measured by a Jarrell-Ash 975 Plasma Atomcomp and was found to be less than 0.08 mol of calcium per mol of CaM. The CaM solution was then lyophilized and dissolved in D$_2$O. The pH was adjusted to 8.0 with KOD or DCI and was not corrected for the deuterium isotope effect. Cation-binding titrations were carried out by adding aliquots (5 μl) of a concentrated stock solution of the desired cation ion to solutions.
of cation-free protein in the NMR tube.  

400 MHz $^1$H NMR spectra were taken on a JEOL GX-400 (Japan) spectrometer operating under a pulse Fourier-transform mode. All spectra were obtained from 512–1024 transients of 90° pulse (4.5 µsec) at 4 sec, and 16 K data points were used for a spectrum width of 5 KHz. Digital resolution was 0.61 Hz/point. Spectra were taken at 24°C, and chemical shifts were measured in parts per million (ppm) from an internal standard of (trimethylsilyl)-propionic acid (TSP).

Resonance signals have been assigned to particular residues of CaM in previous reports (12–14). In this study, the peaks were assigned by these reports and identified by spin-decoupling experiments and the pH titration method, which have been labelled in Fig. 1. Spin-decoupling experiments were performed by time-shared irradiation at a given resonance peak, and the pH titration was performed by adding 4% KOD or 4% DCI solutions to the samples (14).

The aromatic region of the spectrum of CaM without divalent cation and with a 1 molar ratio of various cation/calmodulin in D$_2$O at pH 8.0 and 24°C: [calmodulin] = 0.3 mM. Peaks were assigned in accordance with Seamon (12) and Ikura et al. (14) as follows: (a and a') Tyr-138 δ; (b and b') Tyr-138 ε; (c) Phe-B δ; (d) Phe-A δ; (2 and 2') His-107 H2; (4 and 4') His-107 H4.

Fig. 1. Aromatic regions of the spectrum of calmodulin without divalent cation and with a 1 molar ratio of various cation/calmodulin in D$_2$O at pH 8.0 and 24°C: [calmodulin] = 0.3 mM. Peaks were assigned in accordance with Seamon (12) and Ikura et al. (14) as follows: (a and a') Tyr-138 δ; (b and b') Tyr-138 ε; (c) Phe-B δ; (d) Phe-A δ; (2 and 2') His-107 H2; (4 and 4') His-107 H4.
molar ratio of Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) per CaM at pH 8.0 is shown in Fig. 1. Peaks a and a' of \(\delta\) protons of Tyr-138 were found at 6.65 ppm and 6.34 ppm (Ca\(^{2+}\)), 6.64 ppm and 6.33 ppm (Zn\(^{2+}\)), 6.65 ppm and 6.33 ppm (Cd\(^{2+}\)), 6.63 ppm and 6.33 ppm (Hg\(^{2+}\)), and 6.64 ppm and 6.33 ppm (Mn\(^{2+}\)). Also, peak b' of \(\epsilon\) protons of Tyr-138 was found at 6.55 ppm, 6.54 ppm, 6.54 ppm, 6.54 ppm and 6.55 ppm in Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\) and Mn\(^{2+}\), respectively. Furthermore, the resonances c and d of \(\delta\) protons of one of Phe-B and another Phe-A were found at 6.48 ppm and 6.50 ppm (Ca\(^{2+}\)), 6.48 ppm and 6.48 ppm (Zn\(^{2+}\)), 6.49 ppm and 6.50 ppm (Cd\(^{2+}\)), 6.47 ppm and 6.47 ppm (Hg\(^{2+}\)) and 6.52 ppm and 6.52 ppm (Mn\(^{2+}\)), respectively. However, peaks a, a' and b' were not found at the same points in the range of one molecule Mg\(^{2+}\) spectrum.

Figure 2 shows the lowfield portions of the spectra of CaM in the presence of two molecules of each ion per protein molecule. Two doublet peaks a' and b' were increased in intensity with increasing Ca\(^{2+}\) and Cd\(^{2+}\), and they were unchanged by the Zn\(^{2+}\) additions. Peak c did not change in chemical shift, but showed a decrease in intensity and

![Fig. 2. Aromatic regions of the spectrum of calmodulin without divalent cation and with 2 molar ratio of various cation/calmodulin in D\(_2\)O at pH 8.0 and 24°C.](image-url)
vanished at molar ratio above 2 of Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\) and Mn\(^{2+}\). In concert with the reduction in intensity of peak c, a new peak c' appeared, which grew in intensity with no change in chemical shift produced by cation additions. Peak d shifted downfield sigmoidally with increasing Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\) and Hg\(^{2+}\) contents, but was little changed by Zn\(^{2+}\) and Hg\(^{2+}\).

We have shown that the ions, Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\) and Mn\(^{2+}\), induced spectral changes which were very similar to those seen for Ca\(^{2+}\); however, the Mg\(^{2+}\) induced change was different. This finding is consistent with previous results that the binding patterns of Ca\(^{2+}\) or Mg\(^{2+}\) to CaM were different (12), and the conformation of CaM was nearly the same with Cd\(^{2+}\) ions bound as with Ca\(^{2+}\) bound (15). Also, we have demonstrated that the ethanol-induced sleeping time of mice was increased by treatment with CaCl\(_2\), ZnCl\(_2\), CdCl\(_2\), HgCl\(_2\) or MnCl\(_2\). On the other hand when mice were treated with a CaM antagonist, W-7: [N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide] and the respective cations, the ethanol-induced sleeping time was decreased compared to that of the cation-treated mice without W-7. However, pretreatment of mice with MgCl\(_2\) did not increase the sleeping time. These results suggest that the ethanol-induced sleeping time is enhanced via CaM by Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\) or Mn\(^{2+}\) (8, 9). In the presence of two molecules of cation per protein molecule, individual differences of the cation-induced spectral changes began to appear. The individual differences were more distinct in the presence of three and four cation molecules per protein molecule (our unpublished results). However, we can see three patterns among the cations 1) Ca\(^{2+}\) and Cd\(^{2+}\) induced spectral changes advanced to stage II (fast exchange behavior, see (15)) by the addition of three and four Ca\(^{2+}\) and Cd\(^{2+}\) ions per protein molecule; 2) Zn\(^{2+}\), Hg\(^{2+}\) and Mn\(^{2+}\) induced spectral changes stopped with binding of one cation and did not change significantly by increment of the cation concentration; and 3) the conformation of CaM bound with Mg\(^{2+}\) ions was changed slowly and continuously by increasing Mg\(^{2+}\) concentration.

We suggest that Cd\(^{2+}\) ions bind to all Ca\(^{2+}\)-binding sites of CaM, and this produces a conformational change of CaM as large as Ca\(^{2+}\) ions did; Zn\(^{2+}\), Hg\(^{2+}\) and Mn\(^{2+}\) bind to only the high-affinity Ca\(^{2+}\)-binding site of CaM and induce the large conformational changes that are similar to the 1 molar ratio of Ca\(^{2+}\) induced spectral change; Mg\(^{2+}\) may bind to Ca\(^{2+}\)-binding sites of CaM without inducing the subsequent large change in conformation.

It may be suggested that the differences in conformation of CaM produced by different cations resulted from the different binding constants of the respective cations to CaM. As the next step of investigation, effects of higher concentrations of the cations should be examined to confirm this proposal.

Acknowledgments: We wish to express our thanks to Professor Kunio Hikichi, Hokkaido University, and Dr. Brenda Griffin and Mr. Satoru Numajiri, Alcon Laboratories, Inc., Fort Worth Texas, for reading this manuscript. We would also like to thank Professor Hiroshi Takita, University of Tsukuba, and Professor Kozo Tanabe, Hokkaido University, who kindly gave us encouragement throughout our study. This study was supported in part by a grant (60915003) from the Ministry of Education, Science and Culture of Japan.

References
1 Klee, C.B. and Vanaman, T.C.: Calmodulin. Adv. Protein Chem. 35, 213–321 (1982)
2 Shimizu, T. and Hatano, M.: Effects of metal cations on trifluoperazine-calcimodulin interactions: Induced circular dichroism studies. Biochemistry 23, 6403–6409 (1984)
3 Chao, S.-H., Suzuki, Y., Zysk, J.R. and Cheung, W.Y.: Activation of calmodulin by various metal cations as a function of ionic radius. Mol. Pharmacol. 26, 75–82 (1984)
4 Yamauchi, T. and Fujisawa, H.: Activation of tryptophan 5-monoxygenase by calcium-dependent regulator protein. Biochem. Biophys. Res. Commun. 90, 28–35 (1979)
5 Yamauchi, T. and Fujisawa, H.: Involvement of calmodulin in the Ca\(^{2+}\)-dependent activation of rat brainstem tyrosine 3-monoxygenase. Biochem. Int. 1, 98–104 (1980)
6 Sutoo, D. and Sano, K.: Modulating effects of biogenic amines on calcium and ethanol-induced sleeping time. Alcohol 1, 141–144 (1984)
7 Sutoo, D., Limura, K. and Akiyama, K.: Biogenic
amines synthesis and divalent cations. Folia Pharmacol. Japon. 84, 106P (1984) (in Japanese)

8 Sutoo, D., Akiyama, K. and limura, K.: Effect of calmodulin antagonists on calcium and ethanol-induced sleeping time in mice. Pharmacol. Biochem. Behav. 23, 627–631 (1985)

9 Sutoo, D., Akiyama, K. and limura, K.: The ability of divalent cations to enhance ethanol-induced sleeping time. Alcohol 2 (1985) (in press)

10 Yazawa, M., Sakuma, M. and Yagi, K.: Calmodulins from muscles of marine invertebrates, scallop and sea anemone: Comparison with calmodulins from rabbit skeletal muscle and pig brain. J. Biochem. (Tokyo) 87, 1313–1320 (1980)

11 Calissano, P., Moore, B.W. and Friesen, A.: Effect of calcium ion on S-100, a protein of the nervous system. Biochemistry 8, 4318–4326 (1969)

12 Seamon, K.B.: Calcium- and magnesium-dependent conformational states of calmodulin as determined by nuclear magnetic resonance. Biochemistry 19, 207–215 (1980)

13 Krebs, J. and Carafoli, E.: Influence of Ca²⁺ and trifluoperazine on the structure of calmodulin: A ¹H-nuclear magnetic resonance study. Eur. J. Biochem. 124, 613–627 (1982)

14 Ikura M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M. and Yagi, K.: Nuclear magnetic resonance studies on calmodulin: Calcium-induced conformational change. Biochemistry 22, 2573–2579 (1983)

15 Klevit, R.E., Dalgarno, D.C., Levine, B.A. and Williams, R.J.P.: ¹H-NMR studies of calmodulin: The nature of the Ca²⁺-dependent conformational change. Eur. J. Biochem. 139, 109–114 (1984)