A \(^{1}\text{H}\)-NMR Comparison of Calmodulin Activation by Calcium and by Cadmium

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Abstract—Our previous reports based on pharmacological and histochemical evidence suggest that calcium and cadmium can both activate calmodulin (CaM)-dependent functions. The study reported here was carried out to explain these observations in molecular terms, using \(^{1}\text{H}\)-NMR. Changes in the spectrum of bovine brain CaM induced by 0 to 4 molar equivalents of calcium and cadmium were practically the same. In particular, the chemical shifts and line shape of signals due to Tyr-138, Phe-65, Phe-89 and Met-115 were similarly affected by either ion. In addition, the effects of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, a CaM antagonist) on the phenylalanine aromatic regions, methionine methyl regions and high-field methyl regions of the spectra of both calcium- and cadmium-saturated proteins were practically identical. The effect of W-7 on calcium- and cadmium-saturated CaM was reflected in changes in the signals of Ile-27, Phe-68, Phe-92, Ile-100 and Val-142, as well as Met-71, Met-72, Met-76, Phe-89 and Phe-141. The results show that cadmium binds to all calcium-binding sites of CaM, and induces conformational changes that are as extensive as those brought about by calcium. W-7 also inhibits CaM activation by calcium and cadmium. Combined with our previous toxicological evidence, these results suggest that cadmium binds indiscriminately to CaM and that subsequent activation or modulation of CaM-dependent functions is confused as a result. This may be a mechanism contributing to cadmium poisoning.

Numerous enzyme systems are activated or modulated by calmodulin (CaM) in a calcium-dependent manner. These include detergent-solubilized adenylate cyclase, the erythrocyte calcium-transport system mediated by Ca\(^{2+}\),Mg\(^{2+}\)-ATPase, and the calcium-uptake system in sarcoplasmic reticulum. Smooth muscle myosin light chain kinase is a member of an important class of calcium-dependent kinases which use CaM as a regulatory subunit (1). Furthermore, we have recently demonstrated that the calcium-CaM system activates brain biogenic amine-synthesizing enzymes through a CaM-dependent protein kinase (2, 3). Thus central calcium can reduce blood pressure (4) and modulate animal behavior through a CaM-dependent biogenic amine-synthesizing system (2).

It has also been reported that the CaM-dependent activities of some of these systems were promoted by cadmium as well as calcium. For example, phosphodiesterase activity is enhanced by cadmium via a CaM dependent mechanism (5). Ethanol-induced sleeping time in mice (6) and brain catecholamine levels in mice (7) are increased by cadmium via CaM. Furthermore, we have used \(^{1}\text{H}\)-nuclear magnetic resonance (NMR) spectroscopy to demonstrate that Cd\(^{2+}\) binds to all the Ca\(^{2+}\)-binding sites of CaM, and induces as considerable a conformational change in CaM as Ca\(^{2+}\) (8–10). Based on this evidence, we have suggested a mechanism of cadmium poisoning in which cadmium...
confuses the activation of catecholamine-synthesizing and other enzymes by CaM-dependent systems, thereby disturbing many functions in the organism (11). We also confirmed molecularly in this study whether or not calcium- and cadmium-activated CaM are similarly affected by a CaM antagonist. For this purpose, the effects of Ca$^{2+}$ and Cd$^{2+}$ on the $^1$H-NMR spectrum of CaM were re-examined, and the effect of N-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide (W-7) on the cadmium-induced conformational change of CaM was compared with that on the calcium-induced change of CaM.

**Materials and Methods**

CaM was prepared from bovine brain (donated by Mr. K. Shibata of the Yamagata Local Meat Distribution Center) by a modification of the procedure of Yazawa et al. (12). The protein was judged to be at least 95% pure by its electrophoresis pattern on 7.5% polyacrylamide gel with a Tris-glycine continuous buffer system (13).

The calcium-free protein was prepared by dialyzing 5.0 ml CaM (4 mg/ml) for 24 hr against 1 liter of 1 mM ethylenediaminetetraacetic acid (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 0.5 nM. The Ca$^{2+}$ content of the CaM solution was measured with a Jarrell-Ash 975 Plasma Atom Comp and was found to be less than 0.06 mol Ca$^{2+}$ per mol CaM, and no Cd$^{2+}$ was detected.

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.

Spectra of CaM (0.3 mM) were acquired in the presence of 0 to 4 mole equivalents of both Ca$^{2+}$ and Cd$^{2+}$; these cations bind with a $K_d$ of ca. 10$^{-6}$ M (1.14). Up to three equivalents of W-7, which bind with a $K_d$ of ca. 10$^{-6}$ M (15), were then titrated (5 𝜇l aliquots) from a concentrated stock solution into the metalion saturated CaM.

400 MHz $^1$H-NMR spectra were acquired on a JEOL GX-400 (Japan) spectrometer operating in the pulsed Fourier-transform mode. All spectra correspond to 512 to 1024 transients, obtained using 90° pulses (4.5 μsec) and a 4 sec repetition rate. 16 K data points were used to define a spectral width of 5 kHz, resulting in a digital resolution of 0.61 Hz/point. Spectra were run at 24°C, and chemical shifts were measured in parts per million (ppm) from an internal standard of (trimethylsilyl) propionic acid.

**Results**

**Titration of CaM with calcium or cadmium:** The aromatic and aliphatic regions of the $^1$H-NMR spectrum of CaM are profoundly affected by the addition of Ca$^{2+}$ or Cd$^{2+}$, in agreement with previous reports (8-10, 16-18). Calcium- and cadmium-induced spectral changes were practically the same.

Figure 1 shows the aromatic region of the spectrum of CaM in the presence of 0-4 equivalents of Ca$^{2+}$ (left) and Cd$^{2+}$ (right). Peaks a' and b' (corresponding to the δ and ε protons of Tyr-138) in the Ca$^{2+}$-bound spectrum occur at 6.34 and 6.55 ppm, respectively, compared to 6.33 and 6.54 ppm in the Cd$^{2+}$-bound spectrum. These resonances increase in intensity between molar ratios of Ca$^{2+}$ and Cd$^{2+}$ of 0 and 2, but do not show any change in chemical shift in the course of either addition. Peaks c (Phe-65 δ protons) and d (Phe-16 δ protons) shift downfield sigmoidally with increasing Ca$^{2+}$ and Cd$^{2+}$ content. A new peak e' (Phe-89 δ protons) appears, which increases in intensity with no change in chemical shift during the addition of up to 2 molar ratios of Ca$^{2+}$ and Cd$^{2+}$.

The aliphatic region of the spectrum of CaM under identical conditions of calcium and cadmium concentration is shown in Fig. 2. The single sharp peak due to the ε-methyl protons of the ε-trimethyllysine (Tml)-115 residue appears at 3.13 ppm in the cation-free CaM, and it reduces in intensity as Ca$^{2+}$ is added. At molar ratios of Ca$^{2+}$ to CaM of one, two peaks of nearly equal intensity are observed. At molar ratios of 2 or more, the resonance at 3.13 ppm is absent, and the higher field resonance is invariant. The Cd$^{2+}$-induced changes in Tml-115 residues are very similar to those seen for Ca$^{2+}$.

In many other respects, Cd$^{2+}$- and Ca$^{2+}$-
Fig. 1. Spectra corresponding to 0–4 equivalents of Ca\(^{2+}\), and of Cd\(^{2+}\), of the aromatic region of bovine brain CaM, acquired at 400 MHz in D\(_2\)O at pH 8.0 and 24 °C. [CaM]=0.3 mM. Peaks are assigned in accordance with refs. 16–18, 21: (a and a') Tyr-138 \(\delta\), (b and b') Tyr-138 \(\epsilon\), (c) Phe-65 \(\delta\), (d) Phe-16 \(\delta\), (e and e') Phe-89 \(\delta\), (f) Tyr-99 \(\epsilon\), (g and g') Tyr-99 \(\delta\), (2 and 2') His-107 H2, (4 and 4') His-107 H4. The label numbers A1–A14 correspond to those in Figs. 3 and 4.

Fig. 2. Spectra corresponding to 0–4 equivalents of Ca\(^{2+}\), and of Cd\(^{2+}\), of the aliphatic region of bovine brain CaM, acquired at 400 MHz in D\(_2\)O at pH 8.0 and 24 °C. [CaM]=0.3 mM. The methionine methyl resonances, the high-field methyl resonances and \(\varepsilon\)-methyl resonances of Tml-115 are labelled M1–M9, H1–H9 and Tml, respectively. Assignments are based on refs. 19 and 20, as follows: (H2) Ile-100 \(\delta\), Ile-27 \(\delta\); (H4) Ile-100 \(\gamma\); (H6) Val-142 \(\gamma\); (H8) Leu-116 \(\delta\), \(\delta\'). In addition, M1, M2 or M9 may ascribed to Met-71, Met-72 or Met-76, according to Kievit et al. (21). The label numbers correspond to those in Figs. 5 and 6.
induced spectral changes are very similar. However, there are some differences. Two singlets 2' and 4' (His-107 H2 and H4) occur in the spectrum of Ca2+-bound CaM, although they are not observed at the same chemical shift in the Cd2+-bound protein. Krebs and Carafoli have noted the disappearance of the H2 signal independent of conformational changes in CaM; these signals were however apparent at the same chemical shift in 90 MHz spectra (17). Also, at 2:1 molar ratios of each ion to CaM, peak b' of the Cd2+-bound form is increased in intensity relative to the corresponding peak of the Ca2+-bound species. This is probably due to the fact that peak d overlaps peak b' to a greater extent in the presence of Cd2+ than Ca2+.  

**Titration of Ca2+- and Cd2+-saturated CaM with W-7, aromatic spectral changes:** The appearance of the Ca2+-bound and Cd2+-bound protein aromatic spectrum during the course of the W-7 titration is shown in Fig. 3. The chemical shifts of some of the phenylalanine, tyrosine and histidine signals of CaM are plotted against W-7 concentration in Fig. 4. Previous assignments of some phenylalanine signals (19, 20) have been used in this study, and they are shown in Fig. 3. W-7 affects the aromatic regions of the spectra of Ca2+- and Cd2+-saturated CaM in very similar fashions. In particular, as shown in Figs. 3 and 4, W-7 affects the signals due to the δ and ε protons of Phe-68 and the δ protons of Phe-92, but not the δ and ε protons of Phe-65. Also affected signals are the ε protons of Phe-16, the δ and ε protons of Phe-89, and the δ and ε protons of Phe-141, but not the δ and ε protons of Phe-16, the δ protons of Phe-89 or the ε proton of Phe-141. Tyr-99 δ may also be affected because its resonance contributes to peak A5. The resonances of the δ and ε protons of Tyr-138 and ε protons of Tyr-99 are also unaffected. W-7 also perturbed the H2 and

![Fig. 3](image-url)
H4 resonances of His-107 of Ca\textsuperscript{2+}-bound CaM, but the corresponding changes were impossible to observe in the Cd\textsuperscript{2+}-bound protein.

Titration of Ca\textsuperscript{2+} and Cd\textsuperscript{2+}-saturated CaM with W-7, aliphatic spectral changes: The aliphatic region of the spectrum of CaM saturated with Ca\textsuperscript{2+} or Cd\textsuperscript{2+}, during the course of the W-7 titration is shown in Fig. 5, and the chemical shifts of the methionine and high-field shifted methyl signals are plotted against W-7 concentration in Fig. 6. Previous assignments in the high-field shifted methyl group region (19, 20) and methionine methyl region (21) have been used here, and they are indicated in the legend to Fig. 2.

W-7 exerts very similar effects in the aliphatic regions of the spectra of CaM fully bound with either Ca\textsuperscript{2+} or Cd\textsuperscript{2+}. In particular, as shown in Figs. 5 and 6, Ile-27 δ, Ile-100 δ and γ and Val-142 γ′ methyl groups are affected, as well as, probably Met-71, Met-72 and Met-76, and, to a smaller extent, the Leu-116 δ, δ′ protons. Although H5, H7, H9, M5 and M7 shift upfield, these peaks are unassigned. Tml-115 remains unperturbed, and M3 becomes unobservable due to overlap.

Fig. 4. Chemical shifts of aromatic resonances of calcium-saturated, and cadmium-saturated, CaM plotted against protein : W-7 concentration ratio. Labels of peaks are indicated in Fig. 3.

Discussion

Biogenic amines such as dopamine, noradrenaline and serotonin control many neuronal functions. Recently, we have suggested on behavioral and histochemical grounds that Ca\textsuperscript{2+} acting via CaM can activate the biogenic amine-synthesizing enzymes, tyrosine hydroxylase and tryptophan hydroxylase of mice (2, 3). These CaM-dependent biogenic amine-synthesizing systems are also activated in a similar manner by Cd\textsuperscript{2+}, but not by Mg\textsuperscript{2+} (6). For this reason, the conformational changes of CaM induced by Ca\textsuperscript{2+}, Cd\textsuperscript{2+} and Mg\textsuperscript{2+} were compared by ¹H-NMR (8–10). These studies suggest that Cd\textsuperscript{2+} binds to all Ca\textsuperscript{2+}-binding sites and induces a large conformational change similar to that induced by Ca\textsuperscript{2+}. Although Mg\textsuperscript{2+} binds weakly to the third Ca\textsuperscript{2+}-binding site, it does not interact with the first, second and fourth sites, and does not induce a major conformational change.

Based on this evidence, we propose that the following mechanism may contribute to cadmium poisoning. CaM clearly distinguishes between the two physiologically im-
Fig. 5. The aliphatic region of spectra of calcium- and cadmium-saturated CaM (Ca\(^{2+}\) or Cd\(^{2+}\)/CaM=4.0) during the course of a W-7 titration. The methionine methyl resonances, high-field methyl resonances and \(\varepsilon\)-methyl resonances of Tml-115 are labelled M1–M9, H2–H9 and Tml, respectively, as in Fig. 2. The peaks of W-7 are denoted by asterisks.

Fig. 6. The chemical shifts of methyl resonances of calcium- and cadmium-saturated CaM (Ca\(^{2+}\) or Cd\(^{2+}\)/CaM=4.0) during the W-7 titrations. The methionine methyl resonances and high-field methyl resonances are labelled M1–M9 and H2–H9, respectively, in accordance with Fig. 2.
important metal ions Ca$^{2+}$ and Mg$^{2+}$. On the other hand, Cd$^{2+}$ is naturally present in only negligible quantities in an organism, so no mechanism for distinguishing this ion from Ca$^{2+}$ is required. CaM is no exception in this respect. However, Cd$^{2+}$ is increasingly present as an environmental pollutant and is occasionally ingested by animals and humans. Also, intracellular calcium is meticulously regulated enabling fine control of CaM activity, whereas analogous mechanisms for cadmium homeostasis do not exist. It is thus able to activate Ca$^{2+}$-CaM-dependent functions in an aberrant fashion, as we have previously demonstrated immunohistochemically (7, 11), and in this study, by the use of $^1$H-NMR.

Table 1. A comparison of the effects of W-7 on some of the resonances of calcium- and cadmium-bound calmodulin

| Proton       | Symbol in Figs. | 4Ca-CaM | 4Cd-CaM |
|--------------|-----------------|---------|---------|
| Tyr-138 $\delta$, $\epsilon$ | A1, A2 | - | - |
| Tyr-99 $\epsilon$ | A4 | - | - |
| His-107 H4 | A6 | U | ND |
| Phe-89 $\alpha$, $\zeta$ | A6 | D | D |
| Phe-92 $\delta$ | A10 | U | U |
| His-107 H2 | A14 | U | ND |
| Ile-27 $\delta$, Ile-100 $\delta$ | H2 | D, - | D, - |
| Ile-100 $\gamma$ | H4 | D | D |
| H6 | U | U |
| Val-142 $\gamma'$ | H7 | U, - | U, - |
| Leu-116 $\delta$, $\delta'$ | H8 | D | D |
| H9 | U | U |
| M1 | U | U |
| M2 | D | D |
| M3 | V | V |
| M4 | - | - |
| M5 | U | U |
| M6 | - | - |
| M7 | U | U |
| M8 | V | - |
| M9 | U | U |

Symbols correspond to those in the figures. U: resonance shifts upfield, D: resonance shifts downfield, V: resonance becomes unobservable, ?: resonance only slightly affected, -: unaffected resonance, ND: undetected.

W-7 is a high affinity CaM antagonist that inhibits many of its effects, but which only exerts minor toxic effects on other cellular systems (15). Previous studies (22, 23) by $^1$H-NMR of W-7 binding to Ca$^{2+}$-bound CaM suggest that W-7 interacts with hydrophobic amino acid residues in the vicinity of the calcium-binding sites of domains II, III and IV and influences those. The effects exerted by W-7 appear similar to those of trifluoperazine (a CaM antagonist with other pharmacological properties), D600 (a calcium channel blocker and CaM antagonist) and oxmetidine (a histamine H$_2$ antagonist), whence it may be inferred that they bind to similar regions of the protein. The inhibitory propensities of W-7 also suggest that those residues on CaM with which it interacts are also important in regulating its binding to target enzymes.

This study confirms previous observations of the similarity of the effects of Ca$^{2+}$ and Cd$^{2+}$
on CaM (8–10), and of the effects of W-7 on the Ca^{2+}-saturated protein (22, 23). In addition, we have shown that W-7 affects Cd^{2+}-saturated CaM in a very similar fashion by a detailed comparison of the changes which it induces in the spectrum of each metal-bound form of the protein. Resonances that are comparably affected are due to phenylalanines 68, 89, 92 and 141; methionines 71, 72 and 76; isoleucines 27 and 100; and valine 142. These hydrophobic residues are proposed to be among those at the interface between CaM and its target enzymes.

This evidence suggests that a possible mechanism for cadmium poisoning may reside in the inability of CaM to distinguish between this ion and calcium, resulting in confusion of the natural physiological activities of many enzyme systems by calcium-CaM. This idea is supported by evidence that cadmium-induced testicular damage in mice (24), cadmium-induced microtubule disassembly (25) and cadmium-induced increases in central dopamine synthesis (11) are greatly attenuated by CaM antagonists.

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