^{43}Ca Nuclear Magnetic Resonance of Ca^{2+}–Calmodulin Solutions: Effects of Trifluoperazine and Peptides

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(Received January 15, 1988)

Abstract

By using a conventional FT NMR spectrometer and probe, we first detected ^{43}Ca NMR spectra of the Ca^{2+} (2.9 mM): calmodulin (0.725 mM) (1:1 per binding site) complex in 0.15 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES)–K^+ buffer (pH 7.2). The half-band width of the complex was nearly 160 Hz and the signal of the complex was located at 2.13 ppm (43 Hz) lower field from that of the free Ca^{2+} ion. By adding trifluoperazine, melittin, substance P or glucagon, the half-band widths of the Ca^{2+}–calmodulin complex (1:1 per binding site) were remarkably reduced and the chemical shifts of the complex moved back to the upper field. It is suggested that the Ca^{2+} ion may bind to Ca^{2+} low-affinity sites more tightly in the presence of those effectors than in their absence.

Introduction

^{43}Ca NMR ($I = 7/2$) is very useful to study environments of Ca^{2+}-binding sites in Ca^{2+}-binding proteins [1]. However, using a conventional NMR spectrometer and a conventional probe for liquid samples, ^{43}Ca NMR spectra of Ca^{2+} solutions in the presence of equimolar Ca^{2+}-binding sites in the Ca^{2+}-binding protein are quite difficult to detect because of heavy line broadening of the ^{43}Ca NMR signals of the rapidly exchangeable Ca^{2+} ion. Thus, for the detection of ^{43}Ca NMR spectra of Ca^{2+}–protein solutions, Ca^{2+} solutions in the presence of a very small quantity of the protein had to be used [2].

Calmodulin is an EF-loop Ca^{2+}-binding protein and has four Ca^{2+}-binding sites, two of which are Ca^{2+} high-affinity sites and the others are Ca^{2+} low-affinity sites [1]. In this paper, we present ^{43}Ca NMR spectra of Ca^{2+}–calmodulin solution comprising 1:1 Ca^{2+}:Ca^{2+}-binding sites which were clearly observable in 0.15 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES)–K^+ buffer (pH 7.2). The chemical shifts and half-band widths of ^{43}Ca NMR spectra of the Ca^{2+}–calmodulin (1:1 per binding site) solution were changed by adding trifluoperazine (TFP), a potent calmodulin antagonist, and calmodulin-binding peptides. These experimental conditions will be very useful for further study of ^{43}Ca NMR spectra of Ca^{2+}-binding proteins using conventional NMR spectrometers and probes.

Experimental

Porcine calmodulin was purified to gel-electrophoretical homogeneity as previously described [3]. Thus, calmodulin was purified by precipitation with trichloroacetic acid and by chromatography on phenyl-Sepharose. TFP and HEPES were purchased from Wako Pharmaceutical Co. (Osaka). Melittin, substance P and glucagon were purchased from Sigma Co. ^{43}Co (49.1%) was purchased from the Commissariat à L'Energie Atomique (France) as $CaCO_3$. Other chemicals were of the highest guaranteed grade and were used without further purification.

^{43}Ca NMR spectra were measured on a Bruker CXP-300 FT NMR spectrometer at 20.19 MHz in spinning 10-mm sample tubes with internal 20% D_2O for a frequency lock at 294 K. Typycal spectra of calmodulin solutions consisted of 10^4–10^5 transients using 1000–16000 data points over a 5000-Hz spectral window in quadrature detection mode. The signal/noise ratio was improved by exponential multiplication which introduced 0.5–20 Hz line broadenings. Other NMR conditions were as described previously [2].

Results

The half-band width of the ^{43}Ca NMR spectrum of free Ca^{2+} (2.9 mM) in 0.15 M HEPES–K^+ buffer (pH 7.2) was less than 0.6 Hz, which is essentially the same as that of free Ca^{2+} (2.9 mM) in distilled water (Fig. 1A). Thus, the presence of 0.15 M HEPES–K^+ did not change the movement and/or symmetry of free Ca^{2+} in the aqueous solution and seemed not to hamper the ^{43}Ca NMR spectral study.

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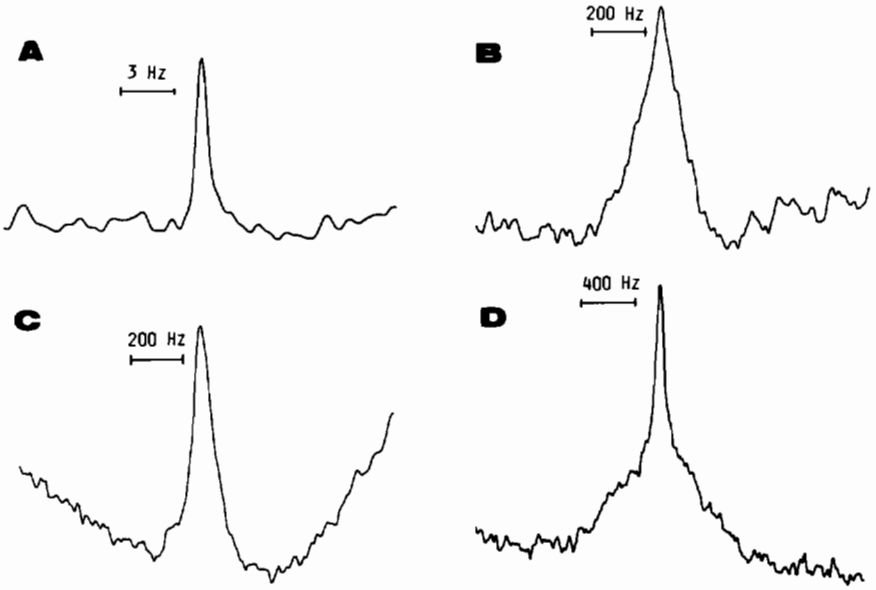

Fig. 1. ^{43}Ca NMR spectra of (A) $^{43}Ca^{2+}$ (2.9 mM), (B) $^{43}Ca^{2+}$ (2.9 mM)–calmodulin (0.725 mM), (C) $^{43}Ca^{2+}$ (2.9 mM)–calmodulin (0.725 mM)–TFP (1.6 mM) and (D) $^{43}Ca^{2+}$ (2.9 mM)–calmodulin (0.725 mM)–melittin (1.0 mM) in 0.15 M HEPES–K^+ buffer (pH 7.2). Number of scans, 2×10^3 (A), 5×10^5 (B), 4×10^5 (C) and 4×10^4 (D); 90° pulse, 80 μs; acquisition time, 4 s(A), 0.12 s(B) (C) (D). Other spectral conditions are described in Experimental.

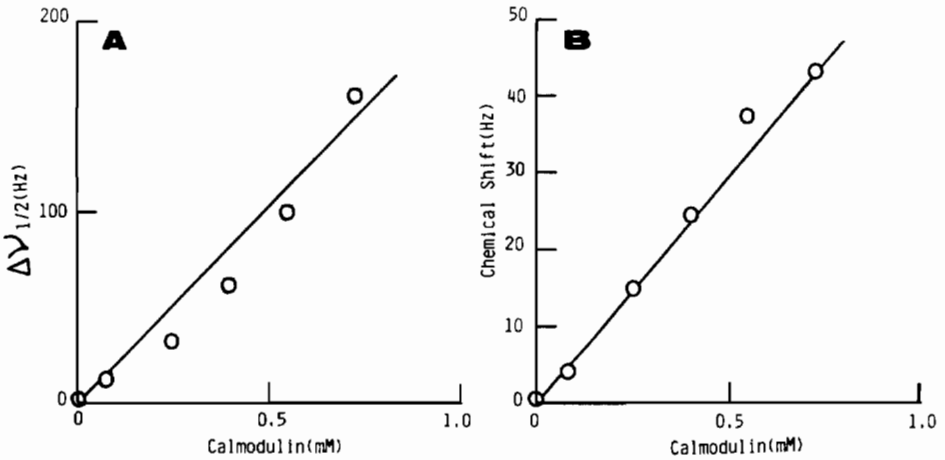

Fig. 2. ^{43}Ca NMR spectral changes in half-band width (Hz) (A) and chemical shift (Hz) (B) for $^{43}Ca^{2+}$ (2.9 mM) on adding calmodulin. Spectral conditions were the same as in Fig. 1.

By adding Ca^{2+}-free calmodulin to 2.9 mM $^{43}Ca^{2+}$ solution, the chemical shift of the ^{43}Ca NMR spectrum moved to lower field by 43 Hz (2.13 ppm) and the half-band width of the ^{43}Ca NMR spectrum increased by up to 160 Hz (Figs. 1B, 2A, 2B). From ^{43}Ca NMR spectral changes caused by adding calmodulin, it was clear that 2.9 mM Ca^{2+}:0.725 mM calmodulin (1:1 per binding site) is observable under these conditions.

By adding TFP, the chemical shift, 43 Hz (2.13 ppm), of the ^{43}Ca NMR spectrum of the Ca^{2+}–calmodulin solution moved back to 10 Hz (0.50 ppm), concomitant with the decrease in the half-band width from 160 to 95 Hz (Figs. 1C, 3A, 3B). The same spectral behavior in ^{43}Ca NMR was observed on adding melittin, a calmodulin-binding peptide, to the Ca^{2+}–calmodulin solutions (Fig. 1D). That is, the chemical shift of the ^{43}Ca NMR spectrum moved back to the upper field and the half-band width of the ^{43}Ca NMR spectrum of the Ca^{2+}–calmodulin solution decreased on adding these peptides (Figs. 3C, 3D). It was noticed that the ^{43}Ca NMR spectrum of the Ca^{2+}–calmodulin–melittin solution consisted of a narrow signal with a half-band width of 90 Hz and a broad signal with a half-band width of 800 Hz. Addition of other calmodulin-binding peptides,

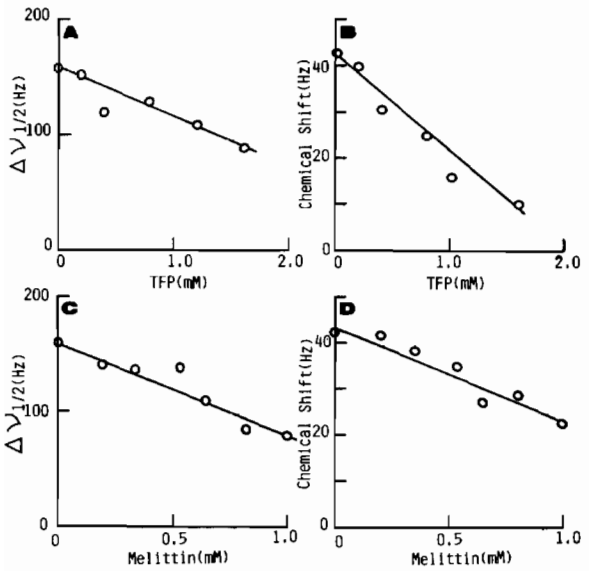

Fig. 3. ^{43}Ca NMR spectral changes for $^{43}Ca^{2+}$ (2.9 mM)–calmodulin (0.725 mM) solutions on adding TFP (A, B) and melittin (C, D). NMR changes of (A) and (C) are expressed in half-band widths (Hz), while those of (B) and (D) are expressed in chemical shifts (Hz).

substance P and glucagon, to the $^{43}Ca^{2+}$–calmodulin (1:1 per binding site) solution also decreased the half-band width of the ^{43}Ca NMR signal by more than 30 Hz and moved the signal back to the upper field by more than 20 Hz (1.0 ppm) (not shown), although precipitates existed in the solution. Addition of polymixin B to the Ca^{2+}–calmodulin solution did not essentially change the ^{43}Ca NMR spectrum of the solution.

Discussion

We observed for the first time ^{43}Ca NMR spectra of the Ca^{2+} solution in the presence of an equimolar concentration (per binding site) of calmodulin by using a conventional NMR spectrometer and its conventional probe. It is well known that calmodulin has four Ca^{2+}-binding sites, two of which are high-affinity sites and the other two are low-affinity sites [1]. Since association constants of Ca^{2+} to two high-affinity sites and two low-affinity sites are 5×10^6 M^{-1} and 5×10^5 M^{-1}, respectively, almost all the Ca^{2+} (2.9 mM) ion in solution must bind to these four Ca^{2+}-binding sites (2.9 mM per Ca^{2+}-binding site). The observed ^{43}Ca NMR signals of the Ca^{2+}–calmodulin solutions may reflect environments of Ca^{2+} low-affinity sites of calmodulin, since K^+ at physiological concentration (0.1–0.2 M) may bind to the Ca^{2+} high-affinity site(s) of calmodulin [2, 4] and thus may antagonize the Ca^{2+} binding to the high-affinity site(s). The chemical shift and half-band width of the Ca^{2+}–calmodulin complex in distilled water or low ionic buffer are 10 ppm and more than 700 Hz, respectively [5] and the signal was ascribed to Ca^{2+} bound at the high-affinity site of calmodulin [5]. The chemical shifts and half-band widths of the complex observed in 0.15 M HEPES–K^+ buffer (pH 7.2) are much smaller than those observed in distilled water or in low ionic buffer. $^{43}Ca^{2+}$ may in part be bound to the sulfonic acid of HEPES, which may apparently decrease binding of $^{43}Ca^{2+}$ to calmodulin. The same effect of HEPES was observed for the ^{67}Zn NMR spectra of Zn^{2+}–calmodulin solutions [2].

TFP tightly binds to calmodulin in the presence of Ca^{2+} [6]. Similarly, some peptides, such as melittin, substance P and glucagon, strongly bind to Ca^{2+}-bound calmodulin with $K_d = 10^{-6}$ M [7, 8]. The ^{43}Ca NMR spectral band-width of $^{43}Ca^{2+}$ in the presence of a small quantity of calmodulin was remarkably reduced by adding TFP [9]. The same spectral change caused by adding TFP was observed for the Ca^{2+}–calmodulin (1:1 per binding site) solution in this study. The chemical shift of the Ca^{2+}–calmodulin (1:1 per binding site) solution moved back to 10 Hz (0.50 ppm) on adding TFP. By adding calmodulin-binding peptides, such as melittin, substance P or glucagon, to the Ca^{2+}–calmodulin (1:1 per binding site) solution, the half-band width decreased and the chemical shift moved back to the upper field in the same way as on adding TFP. Thus, it was found in this study that the environment of probably Ca^{2+} low-affinity site(s) is remarkably changed on adding TFP and other calmodulin-binding peptides. The decrease in the half-band width caused by adding TFP or peptides suggests that Ca^{2+} is more tightly bound in the presence of these effectors.

It is interesting to note that the ^{43}Ca NMR spectrum of the Ca^{2+}–calmodulin–melittin solution consists of a narrow band with a half-band width of 90 Hz and a broad band with a half-band width of 800 Hz. The two ^{43}Ca NMR signals of the Ca^{2+}–calmodulin–melittin solution reflect two Ca^{2+} ions whose environments are different from each other. They probably correspond to a signal from Ca^{2+} at the low-affinity sites and a signal from Ca^{2+} at the high-affinity sites of calmodulin.

The protein structure of calmodulin in 0.15 M K^+ solution may be rather close to that in physiological conditions. Thus the spectrometric data of calmodulin obtained in 0.15 M HEPES–K^+ solution are reliable for a study of the environment of Ca^{2+} in the protein, although interpretation of the results described in this study is rather qualitative.

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