Interaction of trifluoperazine with porcine calmodulin ^{19}F NMR and induced CD spectral studies

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We have used ^{19}F NMR and CD spectra to study interactions of TFP with CaM under various conditions where no aggregation of TFP occurs. It was found that TFP is bound to CaM in the absence of Ca^{2+} with $K_a > 4 \times 10^4$ M in terms of ^{19}F NMR of TFP and that KCl markedly influences the interaction of TFP with CaM. It was also found that TFP causes quite strong induced CD bands corresponding with the absorption bands of the aromatic ring of TFP when TFP is bound to Ca^{2+}–CaM. The induction of the CD bands was time-dependent and was composed of fast and slow phases.

Trifluoperazine *Calmodulin* *^{19}F NMR* *Circular dichroism*

1. INTRODUCTION

Calmodulin (CaM) is a ubiquitous and multifunctional Ca^{2+}-dependent regulatory protein [1,2]. An antipsychotic drug, trifluoperazine (TFP), is strongly bound to CaM in the presence of Ca^{2+} and to be a potent CaM antagonist [3,4]. Microenvironmental changes of CaM caused by TFP binding have been studied with ^{113}Cd NMR [5] and ^1H NMR [6,7] spectra. ^{113}Cd NMR studies have suggested that Ca^{2+}-binding sites of CaM are conformationally changed by adding TFP [5]. From ^1H NMR observations of CaM, it has been suggested that one of the drug binding sites of CaM is located near methionines [6,7]. Comparative ^1H NMR studies for troponin C and CaM have been done as well [8].

We have used ^{19}F NMR and CD spectra to study interactions of TFP with CaM under various conditions where no aggregation of TFP occurs. It was found that TFP is bound to CaM in the absence of Ca^{2+} with $>4 \times 10^4$ M in terms of ^{19}F NMR of TFP and that KCl markedly influences the interaction of TFP with CaM. It was also found that TFP causes quite strong induced CD bands corresponding with the absorption bands of the aromatic ring of TFP when TFP is bound to Ca^{2+}–CaM. The induction of the CD bands was time-dependent and was composed of fast and slow phases.

Abbreviations: CaM, calmodulin; TFP, trifluoperazine; NMR, nuclear magnetic resonance; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; K_d, dissociation constant

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2. MATERIALS AND METHODS

CaM was prepared from porcine brain by a modification of the methods in [9,10]. Briefly our method included precipitation with trifluoroacetic acid, heat treatment and chromatography on phenyl–Sepharose. The purity of the protein was checked by SDS gel electrophoresis and ^1H NMR. TFP was a kind gift from Yoshitomi Pharmaceutical Co. (Osaka).

^{19}F NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 282.3 MHz with external D_2O for the frequency lock. NMR spectra were obtained at 298 ± 0.5 K. Chemical shifts in ppm were referred from those of ^{19}F

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nuclei of TFP (0.5 mM) in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0). Resonances occuring at down-field region were taken as positive in ppm.

The CD and absorption spectra were obtained at 298 ± 0.5 K on a JASCO J-500 spectropolarimeter and JASCO UNIDEC-510 spectrometer, respectively.

pH-Values were strictly adjusted at 7.0 ± 0.3 before the NMR and CD spectral measurements and were again checked after the spectral measurements. NMR spectra were always obtained >40 min after sample preparations to make spectra settle down.

3. RESULTS

3.1. *^{19}F NMR spectra*

^{19}F NMR resonance of 0.5 mM TFP moved to down-field by 0.4 ppm by adding CaM in the presence of 2 mM EGTA (fig.1A,B). The half-band width, 6 Hz, of ^{19}F NMR of TFP was also increased to 29 Hz by adding CaM in the presence of 2 mM EGTA. The spectral change was saturated when equimolar CaM was present in the solution (fig.2A, —△—. Adding 0.2 M KCl to the EGTA–TFP–CaM solution made the resonance shift to higher-field by 0.08 ppm and the half-band width decrease to 12 Hz (fig.1C). ^{19}F NMR titration studies were also done for TFP in 0.2 M KCl–1 mM EGTA (—●—) and 0.2 M KCl–2.2 mM $CaCl_2$–3.9 mM EGTA (—○—) solutions as shown in fig.2A. The spectral changes of the KCl solutions were essentially the same as each other, but were smaller than that (—△—) of the non-KCl solution. The spectral change of TFP in the KCl solution was saturated in the presence of equimolar CaM.

Changes of the chemical shifts and the half-band widths of TFP caused by adding CaM in the presence of excess Ca^{2+} (or in the absence of EGTA) were more remarkable than those in the absence of Ca^{2+} (or in the presence of EGTA). Fig.1D shows ^{19}F NMR of 0.5 mM TFP in the presence of 0.5 mM CaM and 2.2 mM $CaCl_2$. The TFP–CaM solutions with CaM/TFP ratios more than unity offered quite broad ^{19}F NMR bands which were essentially undetectable even after 10^5 transients. The NMR spectral change of the $CaCl_2$ solution was peculiar in that titrating curve on the chemical shift did not form a hyperbolic pattern, but exhibited a trough at 0.2 mM CaM as shown in fig.2B (—●—). ^{19}F NMR band (fig.1E) of TFP–CaM (1 : 1) solution with 0.2 M KCl–2.2 mM $CaCl_2$ was located at 0.05 ppm higher than that of the $CaCl_2$ solution (fig.1D). The spectral change caused by adding CaM was larger in the 0.2 M KCl– 2.2 mM $CaCl_2$ solution than that in the 2.2 mM $CaCl_2$ solution.

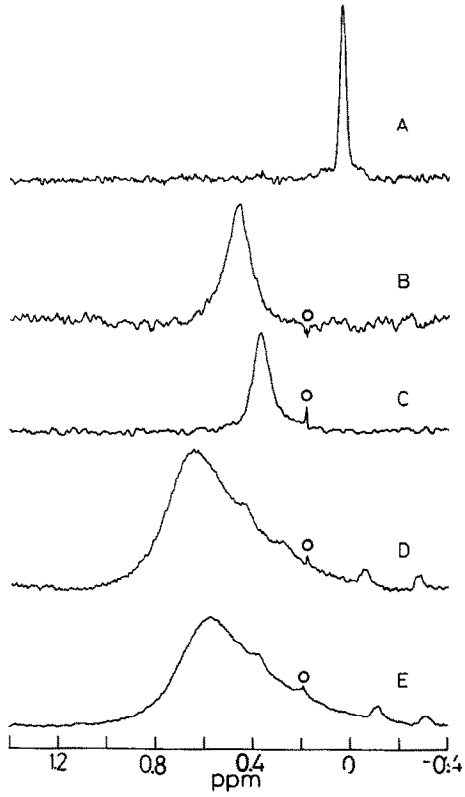

Fig.1. ^{19}F NMR spectra of: (A) 0.5 mM TFP in 0.2 M KCl–2.2 mM $CaCl_2$; (B) 0.5 mM TFP–0.5 mM CaM–1 mM EGTA; (C) 0.5 mM TFP–0.5 mM CaM–1 mM EGTA–0.2 M KCl; (D) 0.5 mM TFP–0.5 mM CaM–2.2 mM $CaCl_2$; (E) 0.5 mM TFP–0.5 mM CaM–2.2 mM $CaCl_2$–0.2 M KCl solutions; pH-values of the solutions were kept at 7.0 ± 0.3; sweep width, 1000 Hz; number of scans, 60 for (A), (B) and (C) or 4 × 10^4 for (D) and (E); line broadening, 1 Hz; pulse width, 10 μs (45° pulse); acquisition time, 1.5 s. The small circles are indicative of center peaks.

3.2. *Induced CD spectra*

CD bands of aromatic compounds are induced at the absorption wavelengths of the achiral molecule by the dissymetric perturbation of the chiral species [11–13]. This phenomenon, induced CD,

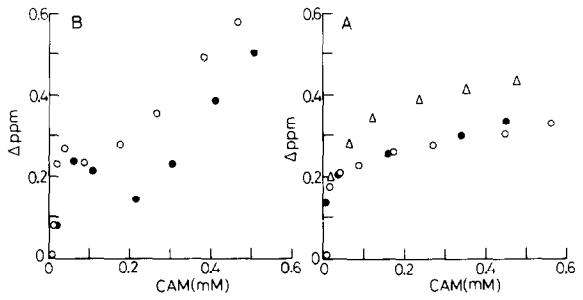

Fig.2. (A) ^{19}F NMR titrations of 0.5 mM TFP *vs* CaM in: 1 mM EGTA (—Δ—); 0.2 M KCl–1 mM EGTA (—●—); 0.2 M KCl–2.2 mM $CaCl_2$–3.9 mM EGTA (—○—) solutions. (B) ^{19}F NMR titrations of 0.5 mM TFP *vs* CaM in 2.2 mM $CaCl_2$ (—●—) and 0.2 M KCl–2.2 mM $CaCl_2$ (—○—) solutions. pH-Values of the solutions were kept at 7.0 ± 0.3. Other spectral conditions were the same as in fig.1.

has been observed at the optical absorption bands of the achiral aromatic molecule which is inserted in the cavity of the host chiral molecule and is also found to be useful for studying the polarization direction of the electronic transition of the achiral aromatic molecule [13]. CD spectra of TFP in the presence of CaM over 250–400 nm were studied under various conditions. TFP itself is achiral and thus no CD band was observable at 254 nm and 303 nm of its absorption peaks. CaM (50 μM)–TFP (100 μM) solution in the presence of 2 mM EGTA exhibited small negative CD band from 250–340 nm (▬ in upper fig.3). Absorption peaks were located at 256 nm and 303 nm. Absorptions which were neither seen for TFP itself nor for CaM itself were seen from 325–340 nm. By adding 0.2 M KCl to the CaM (50 μM)–TFP (100 μM)–EGTA (2 mM) solution, positive CD bands appeared from 250–350 nm (--Δ-- in upper fig.3) with a maximum peak around 280 nm. The absorption peaks at 256 nm and 303 nm were shifted to 254 nm and 302 nm, respectively, by adding KCl (--Δ-- in lower fig.3). A shoulder around 275 nm on the absorption spectrum appeared additionally for the 0.2 M KCl solution. Adding excess $CaCl_2$ (4 mM) to the 2 mM EGTA–0.2 M KCl solution caused a quite large CD trough around 262 nM together with peaks around 293 nm and 315 nm and a trough around 360 nm (—○— in upper fig.3). The absorption bands at 254 nm and 302 nm of the 2 mM EGTA–0.2 M KCl solution were shifted to

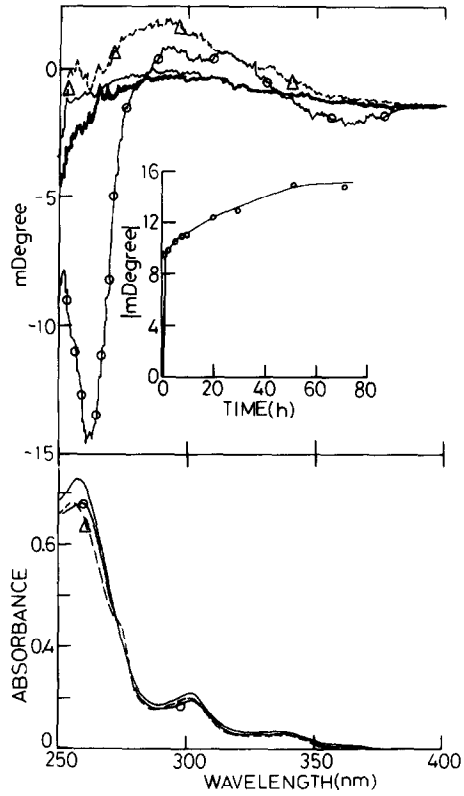

Fig.3. CD (upper) and absorption (lower) spectra of CaM (50 μM)–TFP (100 μM) in the 2 mM EGTA solution (▬ of CD and —— of absorption), CaM (45 μM)–TFP (90 μM) in the 2 mM EGTA–0.2 M KCl (--Δ--) and 2 mM EGTA–0.2 M KCl–4 mM $CaCl_2$ (—○—) solutions. CD spectrum of CaM (45 μM)–TFP (90 μM) in the 0.2 M KCl–2.2 mM $CaCl_2$ solution was essentially the same as that in the 2 mM EGTA–0.2 M KCl–4 mM $CaCl_2$ solutions; pH values were kept at 7.0 ± 0.3; pathlength, 1 cm; time constant, 8 s; scan speed, 10 nm/min; spectral bandwidth, 2 nm; (——) baseline for CD spectra. Inset: time-dependent change in absolute CD magnitudes of CaM (50 μM)–TFP (100 μM) in 0.2 M KCl–2.2 mM $CaCl_2$ solution.

260 nm and 304 nm, respectively, by adding 4 mM $CaCl_2$. The absorption shoulder at 275 nm observed for the 2 mM EGTA–0.2 M KCl solution was not seen for the 4 mM $CaCl_2$–2 mM EGTA–0.2 M KCl solution. The CD trough at 262 nm was time-dependently enlarged and reached the maximum about 50 h after the sample preparation. The inset in fig.3 shows the time-dependent change of the absolute CD magnitude at 262 nm. Small increase of the absorption spectra at 260 nm was also noted

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