Interaction of trifluoperazine with *Tetrahymena* calmodulin

A ^{19}F NMR study

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We have used ^{19}F NMR to study interactions of trifluoperazine (TFP), a potent calmodulin (CaM) antagonist, with *Tetrahymena* calmodulin (*Tet.* CaM). Changes in chemical shift and bandwidth of TFP caused by adding *Tet.* CaM in the presence of excess Ca^{2+} were much smaller than those by adding porcine CaM. The spectral features of the TFP–*Tet.* CaM solution in the presence of excess Ca^{2+} were quite similar to those of the TFP–porcine CaM solution in the absence of Ca^{2+}. The exchange rate of TFP from *Tet.* CaM was estimated to be nearly 20 s^{-1}. The TFP–*Tet.* CaM solution in the absence of Ca^{2+} showed a pronounced pH dependence of the ^{19}F NMR chemical shift, whereas the solution in the presence of excess Ca^{2+} showed a smaller pH dependence. Thus, it was suggested that TFP is located near a hydrophilic region of the *Tet.* CaM molecule in the absence of Ca^{2+}, while TFP is located near a hydrophobic region of the *Tet.* CaM in the presence of excess Ca^{2+}.

Trifluoperazine Tetrahymena *Calmodulin* *^{19}F NMR*

1. INTRODUCTION

*Tet.*CaM presents interesting contrasts to vertebrate CaM [1,2]. For example, *Tet.* CaM has 11 amino acid substitutions and one deletion of an amino acid residue compared with bovine brain CaM [1], is very specific to activate *Tetrahymena* guanylate cyclase [2] and activates bovine brain phosphodiesterase less compared with vertebrate CaM [2]. An antipsychotic drug, TFP, is known to be strongly bound to CaM with $K_d \simeq 10^{-6}$ M in the presence of excess Ca^{2+} and to be a potent CaM antagonist [3–5]. We have found [6] that TFP interacts with Ca^{2+}-free porcine CaM with $K_d \simeq 10^{-5}$ M and that KCl markedly affects the interaction of TFP with porcine CaM in terms of ^{19}F NMR spectra.

We have here examined the interaction of TFP with *Tet.* CaM with ^{19}F NMR of TFP. It was found that TFP is bound to *Tet.* CaM weakly compared with porcine CaM [6] in terms of ^{19}F NMR. In addition, it was suggested that TFP is located near a hydrophilic region of the Ca^{2+}-free *Tet.* CaM molecule, while TFP is located near a hydrophobic region of the Ca^{2+}-bound *Tet.* CaM molecule.

Abbreviations: Tet. CaM, calmodulin purified from *Tetrahymena pyriformis*; CaM, calmodulin; TFP, trifluoperazine; CD, circular dichroism; SIS, solvent-induced shift

2. MATERIALS AND METHODS

Tet. CaM was purified as in [1,2,7]. The *Tet.* CaM preparation was homogeneous upon polyacrylamide gel electrophoresis. KCl, $CaCl_2$ and EGTA of guaranteed grade were used without further purification. TFP was kindly supplied from Yoshitomi Pharmaceutical Co., Osaka. Solu-

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tions for NMR measurements consisted of 0.2 M KCl and 2.2 mM $CaCl_2$ unless otherwise noted and were strictly adjusted to appropriate pH with 1 M KOH or 1 M HCl before NMR measurements.

^{19}F and ^{1}H NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 282.3 and 300.1 MHz, respectively, as in [6]. Chemical shifts in ppm were referred from those of ^{19}F nuclei of TFP (0.5 mM) in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0 ± 0.1). Resonances occurring in the down-field region were taken as positive in ppm. The longitudinal relaxation rate, T_1, was determined by the inversion recovery technique (180°–τ–90° pulse sequence).

3. RESULTS

We obtained 300 MHz ^{1}H NMR spectra of purified *Tet.* CaM for the first time to determine the effect of TFP addition on the structure of *Tet.* CaM. On the whole, the ^{1}H NMR spectrum of *Tet.* CaM in the presence of Ca^{2+} was similar to those of bovine CaM [8,9] or porcine CaM [10]. The main points we noticed on the ^{1}H NMR spectrum (fig.1A) of *Tet.* CaM-Ca^{2+} are:

(i) Peaks ascribed to Tyr-99 at 6.76 and 7.29 ppm were practically unobservable in accordance with the amino acid analysis [1], which showed a substitution of Tyr-99 by Leu-99;
(ii) The H-2 proton of His-135 was observed at 8.45 ppm in addition to the H-2 proton of His-107 at 7.75 ppm, which was observed for bovine and porcine CaM [8–10].

By adding TFP to *Tet.* CaM-Ca^{2+}, the ^{1}H NMR spectrum of *Tet.* CaM was changed essentially similarly to bovine and porcine CaM [9,11] as shown in fig.1B. For example,

(i) The methyl peak at 0.86 ppm began to split to a doublet at [TFP]/[*Tet.* CaM] ≃ 1;
(ii) The well resolved methionine signals around 2 ppm became broader;
(iii) A peak at 2.56 ppm appeared and was comparable to that at 2.74 ppm;

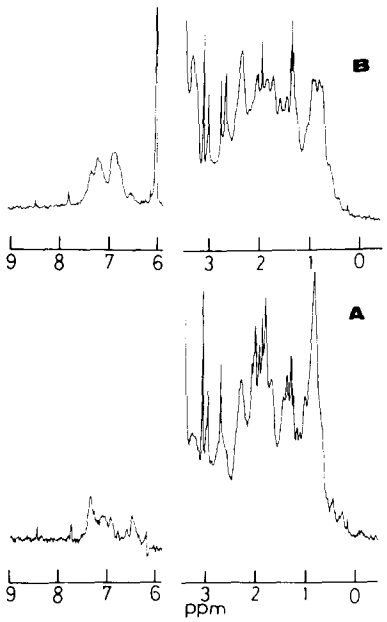

Fig.1. 300 MHz ^{1}H NMR spectra of 0.5 mM *Tet.* CAM (A) and 0.5 mM *Tet.* CaM–1.1 mM TFP (B) in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0 ± 0.1). A strong peak at 6.05 ppm in (B) is ascribable to TFP. Sweep width, 10000 Hz; data points, 16 K; number of scans, 3000; pulse width, 5 μs (70° pulse); recycle time, 2.1 s; exponential line broadening, 2 Hz; sample temperature, 298 ± 0.5 K; quadrature detection mode.

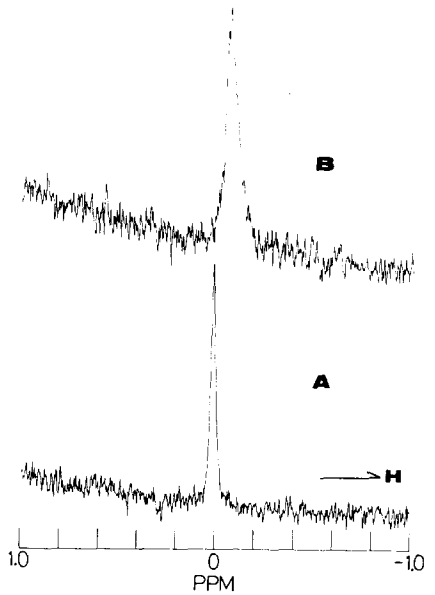

Fig.2. ^{19}F NMR spectra of 0.5 mM TFP (A) and 0.5 mM TFP–0.281 mM *Tet.* CaM (B) in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0 ± 0.1). Sweep width, 2000 Hz; data points, 16 K; number of scans, 200 (A) or 400 (B); pulse width, 20 μs (90° pulse), recycle time, 10 s; exponential line broadening, 1 Hz; sample temperature, 298 ± 0.5 K; quadrature detection mode.

Table 1

^{19}F NMR spectra of TFP and the TFP–*Tet.* CaM complex

	Chemical shift (ppm)	Half bandwidth (Hz)	T_1 (s)	SIS (ppm)
TFP (0.5 mM)	0	6.5	1.56 ± 0.02	0.1
TFP (0.5 mM)–*Tet.* CaM (0.281 mM)	−0.07	18.2	0.58 ± 0.02[a]	<0.01

[a] T_1 value of TFP–*Tet.* CaM complex was estimated from $1/T_1 = 1/T_{TFP} - 1/T_{app}$ where T_{TFP} is T_1 of free TFP in aqueous solution and T_{app} is apparent T_1 (0.42 ± 0.02 s) obtained for the TFP–*Tet.* CaM complex in aqueous solution

(iv) The broad peak at 3.28 ppm increased;
(v) The aromatic peaks from 6.6 to 7.4 ppm were simplified to two broad peaks as for bovine CaM [9].

It should be noted that ^{1}H NMR spectral changes of *Tet.* CaM caused by adding TFP were nearly saturated at [TFP]/[*Tet.* CaM] ≃ 2 under our experimental conditions, similarly to those observed for bovine and porcine CaM [9,11]. Detailed analyses of ^{1}H NMR of *Tet.* CaM will be published elsewhere.

^{19}F NMR changes of TFP caused by adding *Tet.* CaM in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0 ± 0.1) were studied. Changes of chemical shifts caused by adding *Tet.* CaM were less than 0.1 ppm and the bandwidth of TFP was increased only 3-fold by

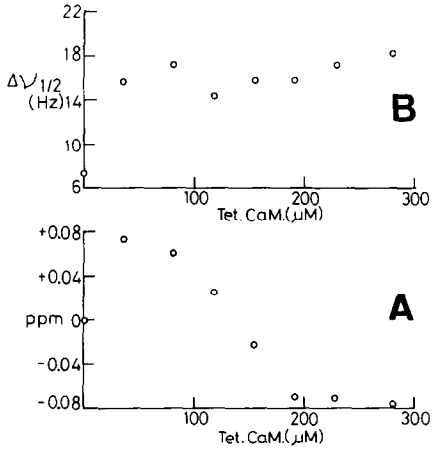

Fig.3. ^{19}F NMR spectral changes of 0.5 mM TFP (pH 7.0 ± 0.1) caused by adding *Tet.* CaM. (A) Spectral changes in terms of chemical shift in ppm, (B) spectral changes in terms of the half-bandwidth in Hz. The solution consisted of 0.2 M KCl and 2.2 mM $CaCl_2$. Other experimental conditions were the same as in fig.2.

adding *Tet.* CaM (fig.2, table 1). The ^{19}F NMR changes of TFP (0.5 mM) in chemical shift or bandwidth were not monophasic as observed for porcine CaM, and were saturated by adding 0.20–0.25 mM *Tet.* CaM (fig.3). The solvent-induced shift [12] (0.1 ppm) of free 0.5 mM TFP was decreased to less than 0.01 ppm by adding 0.28 mM *Tet.* CaM (table 1), suggesting that TFP is fully bound to *Tet.* CaM under these conditions. In accordance with the 3-fold increase in bandwidth of free TFP on addition of *Tet.* CaM, the T_1 value (1.56 s) of free TFP decreased to 0.58 s on addition of *Tet.* CaM. The chemical shift and bandwidth of TFP–*Tet.* CaM solution are very different from those observed for TFP–porcine CaM solution [6]. The chemical shift and bandwidth of TFP–porcine CaM solution were more than 0.6 ppm and 110 Hz, respectively [6]. Thus, it seems likely that the exchange rate of TFP from *Tet.* CaM is quite different from that of TFP from porcine CaM. By adding more than 4 mM EGTA, the chemical shift of TFP–*Tet.* CaM solution moved further to high field by 0.08 ppm.

Since the bandwidth of *Tet.* CaM solution was not very broad, it was feasible to study the pH dependence of the ^{19}F NMR chemical shifts of TFP–*Tet.* CaM solution. Fig.4 shows the pH dependence of the ^{19}F NMR chemical shifts of free TFP, and TFP–*Tet.* CaM solutions in the presence and absence of Ca^{2+}. The TFP–*Tet.* CaM solution in the absence of Ca^{2+} (in the presence of excess EGTA) showed a pronounced pH dependence, whereas that in the presence of excess Ca^{2+} showed a smaller pH dependence. TFP itself scarcely showed a pH dependence. The presence of excess EGTA did not affect the pH dependence of free TFP.

The induced CD spectrum of TFP

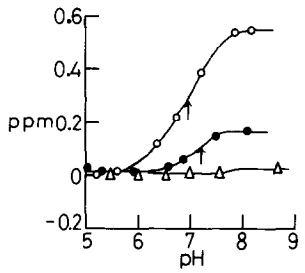

Fig.4. ^{19}F NMR spectral changes of 0.5 mM TFP (△), 0.5 mM TFP–0.25 mM *Tet.* CaM (●) and 0.5 mM TFP–0.25 mM *Tet.* CaM in the presence of 4.87 mM EGTA (○) as functions of pH. The solutions consisted of 0.2 M KCl and 2.2 mM $CaCl_2$. Other experimental conditions were the same as in fig.2. The chemical shifts are normalized to those at acidic regions.

(100 μM)–*Tet.* CaM (50 μM) in 0.2 M KCl–2.2 mM $CaCl_2$ (pH 7.0 ± 0.1) solution was obtained. The spectral feature (not shown) was similar to that described for porcine CaM [6]. The CD magnitude, however, was nearly 8 mdeg, which is much smaller than that (≃15 mdeg) observed for TFP (100 μM)–porcine CaM (50 μM) solution.

4. DISCUSSION

The ^{19}F NMR results on the TFP–*Tet.* CaM solution in 0.2 M KCl–2.2 mM $CaCl_2$ were very different from those observed for TFP–porcine CaM solution. From the ^{1}H NMR changes of *Tet.* CaM caused by adding TFP and the ^{19}F NMR changes of TFP on addition of *Tet.* CaM, it seems likely that 2 molecules of TFP are bound to *Tet.* CaM under our experimental conditions. The ^{19}F NMR spectral feature of TFP–*Tet.* CaM solution in 0.2 M KCl–2.2 mM $CaCl_2$ was similar to that of TFP–porcine CaM solution in the absence of Ca^{2+} (in the presence of excess EGTA [6]). The exchange rate of TFP in TFP–porcine CaM in the absence of Ca^{2+} would be nearly 40 s^{-1} and that in the TFP–*Tet.* CaM in the presence of excess Ca^{2+} almost 20 s^{-1}. These exchange rates are quite slower than that (≃10^2 s^{-1}) [11] for TFP–bovine CaM in the presence of excess Ca^{2+}. Thus, it is suggested that TFP binding to *Tet.* CaM in the presence of excess Ca^{2+} will be different from that to porcine or bovine CaM. This is in accordance with fluorescence results on *Tet.* CaM [13] that the surface of the *Tet.* CaM–Ca^{2+} molecule is less hydrophobic than that of bovine CaM. From ^{1}H NMR studies, it was suggested [9,11] that methionine is located near the TFP-binding site of bovine CaM. The Met-71 of bovine CaM is substituted by Leu-71 in *Tet.* CaM [1]. Thus, this substitution may explain the difference in the TFP interaction between bovine CaM and *Tet.* CaM.

The ^{19}F NMR spectrum of free TFP itself scarcely moved as the pH was changed and thus the ^{19}F NMR changes of TFP–*Tet.* CaM solutions, depending on pH, are not due to protonation (or deprotonation) of the solvent. We found previously that TFP interacts weakly with porcine CaM even in the presence of excess EGTA [6]. TFP will also bind weakly to *Tet.* CaM in the presence of excess EGTA. Since the dissociation of Ca^{2+} from TFP–*Tet.* CaM takes place below pH 6.5 [3,14], it is unlikely that the pH dependence observed for TFP–*Tet.* CaM solutions is related to Ca^{2+} dissociation from *Tet.* CaM. ^{19}F NMR changes observed for TFP–*Tet.* CaM solutions as a function of pH are decidedly associated with protonation (or deprotonation) of the protein surface with which TFP is interacting. It is suggested that TFP is bound to a hydrophobic site on the protein surface in the presence of excess Ca^{2+}, whereas in the presence of excess EGTA TFP will bind to another hydrophilic binding site of *Tet.* CaM. Since the apparent pK_a (~7.0) of TFP–*Tet.* CaM complex in the presence of EGTA is that of imidazole (5.6–7.0) [15], it is likely that histidine imidazole is in close proximity to TFP in the complex. The significance of the hydrophobic interactions of bovine CaM with CaM-dependent enzymes has been suggested [16,17]. The inhibition of *Tetrahymena* Ca^{2+}-dependent functions by TFP [18] would be caused by hydrophobic interaction of TFP with the protein. It was suggested from equilibrium dialysis study that nearly half of the TFP molecules in TFP–bovine CaM (2:1) complex is dissociated from bovine CaM in the presence of Ca^{2+} at pH > 7.5 [3]. The ^{19}F NMR change of TFP–*Tet.* CaM in the presence of Ca^{2+} at pH > 7.5 may thus be due to TFP still bound to *Tet.* CaM since free TFP is hardly affected by the pH of the solution and the dissociation of TFP from *Tet.* CaM is expected to produce a 0.07 ppm down-field shift of the ^{19}F NMR of TFP (fig.2,3; table 1).

In conclusion, it was found from ^{19}F NMR studies of TFP bound to *Tet.* CaM that:

(i) TFP interacts with *Tet.* CaM in a way different from that of TFP with porcine CaM; and

(ii) TFP is located near a hydrophilic site of the *Tet.* CaM molecule in the absence of Ca^{2+}, while TFP is located near a hydrophobic site of the *Tet.* CaM molecule in the presence of excess Ca^{2+}.

Since ^{43}Ca NMR results obtained on *Tet.* CaM–Ca^{2+} solution were not very different from those of bovine CaM [14], the high utility of ^{19}F NMR for studying the drug–protein interactions should be emphasized.

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