Effects of Metal Cations on Trifluoperazine–Calmodulin Interactions: Induced Circular Dichroism Studies†

Toru Shimizu* and Masahiro Hatano*

ABSTRACT: Interactions of trifluoperazine (TFP) with porcine brain calmodulin (CaM) were studied under various conditions with induced circular dichroism (CD) spectra. The TFP (90 μM)–CaM (45 μM) solution in distilled water showed a large positive CD band (7 mdeg) around 265 nm corresponding to the absorption peak of TFP. The positive peak around 265 nm of the TFP–CaM solution was vanished by adding alkaline metal cations. Thus, it was suggested that CaM does not necessitate Ca^{2+} for TFP binding and that negatively charged groups such as carboxylate groups in CaM contribute to the CaM–TFP interaction as suggested by Gariépy and Hodges [Gariépy, J., & Hodges, R. S. (1983) *Biochemistry 22*, 1586–1594] by homology of troponin C. By adding Ca^{2+} to the TFP (90 μM)–CaM (45 μM) complex in 0.2 M KCl solution, a large negative CD band (−15 mdeg) was induced around 260 nm corresponding to the absorption peak of TFP. Mn^{2+}, Cd^{2+}, and Zn^{2+} also induced the large negative CD bands (12–13 mdeg) for the TFP (90 μM)–CaM (45 μM) complex in 0.2 M KCl solutions. It was suggested that these bivalent metal cations cause a conformational change of CaM and alter the characteristics of the specific CaM–TFP interaction present in the absence of Ca^{2+}. Co^{2+} and alkaline earth metal cations such as Mg^{2+}, Sr^{2+}, and Ba^{2+} caused only a small negative CD band (2–5 mdeg) of the TFP–CaM complex in 0.2 M KCl solution. However, the magnitudes of the negative CD bands around 260 nm caused by adding Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Sr^{2+}, and Ba^{2+} to the TFP–CaM complex in distilled water were much larger than those in 0.2 M KCl solution. Thus, competitive binding of alkaline monovalent cations with the bivalent cations was implied. Other phenothiazines such as chlorpromazine, fluphenazine, and propericiazine also induced large CD bands under the same conditions.

Calmodulin (CaM)[1] is a ubiquitous and multifunctional Ca^{2+}-dependent regulatory protein (Cheung, 1980; Means et al., 1982; Kakiuchi et al., 1982). The significance of hydrophobic interactions of bovine CaM with CaM-dependent enzymes has been suggested (LaPorte et al., 1980; Tanaka & Hidaka, 1980). An antipsychotic phenothiazine, trifluoperazine (TFP), is strongly bound to CaM probably through hydrophobic interaction in the presence of excess Ca^{2+} and is a potent CaM antagonist (Levin & Weiss, 1977; Bromstrom et al., 1982; Roufogalis, 1982). TFP is believed to be bound to the CaM molecule in the same way as CaM-dependent enzymes. Thus, it will be a good model to investigate the interaction of TFP with CaM by a useful method to know the structure–function relationship of CaM in cell functions. Microenvironmental changes of CaM caused by TFP binding have been studied with ^{113}Cd (Andersson et al., 1983; Thulin et al., 1984) and ^{1}H NMR spectra (Klevit et al., 1981; Krebs & Carafoli, 1982). It has been suggested from chemical modification studies (Walsh & Stevens, 1978) and ^{1}H NMR studies (Klevit et al., 1981; Krebs & Carafoli, 1982) that methionine in CaM may in part contribute to the TFP–CaM interaction. ^{19}F NMR spectra of TFP–CaM solutions were also obtained for studying the interaction of TFP with CaM (Shimizu & Hatano, 1983; Shimizu et al., 1984).

Interactions of bivalent metal ions other than Ca^{2+} with CaM have been investigated in relation to the functions of CaM (Wolff et al., 1977; Dedman et al., 1977; Crouch & Klee, 1980; Haiech et al., 1981). Bivalent metal cations such as Zn^{2+} and Mg^{2+} affect ^{43}Ca NMR spectra of Ca^{2+}–CaM solutions (Shimizu, T., et al., 1982; Andersson et al., 1982). Correlation to bivalent metal toxicity with CaM inhibition was also studied (Cox & Harrison, 1983). The presence of KCl markedly changes the binding behavior of Ca^{2+} to Ca^{2+}-free CaM (Wolff et al., 1977; Dedman et al., 1977; Crouch & Klee, 1980; Haiech et al., 1981). It was suggested from the ^{19}F NMR study that KCl markedly influences the interaction of TFP with CaM (Shimizu & Hatano, 1983). The backbone structure of CaM is remarkably changed in terms of CD spectra in the far-UV region (Walsh & Stevens, 1978; Wolff et al., 1977; Dedman et al., 1977; Crouch & Klee, 1980; Liu & Cheung, 1976; Klee, 1977) by adding excess Ca^{2+}, Mg^{2+}, or Mn^{2+} to Ca^{2+}-free CaM. The near-UV CD bands were also markedly influenced by adding excess Ca^{2+} or KCl to CaM (Wolff et al., 1977; Crouch & Klee, 1980; Klee, 1977). However, the far-UV CD spectra of Ca^{2+}-bound CaM are not markedly changed by adding TFP as will be mentioned later. A change of the near-UV CD band caused by adding TFP to Ca^{2+}-bound CaM cannot be monitored due to the intense absorption bands of TFP itself.

When the achiral aromatic molecule is inserted in the cavity of the host chiral molecule, CD bands of the aromatic compound are induced at the absorption wavelengths of the achiral molecule by the dissymmetric perturbation of the chiral species (Hatano et al., 1973; Murakami & Hatano, 1975; Shimizu, H., et al., 1982). This phenomenon, induced CD, is found to be quite useful for studying the polarization direction of the electronic transition of the achiral aromatic molecule (Shimizu, H., et al., 1982). We previously found that TFP gives rise to a large induced CD band at the absorption peak of TFP when TFP is added to porcine CaM solution containing excess Ca^{2+} and 0.2 M KCl (Shimizu & Hatano, 1983). It was felt worthwhile to investigate induced CD bands of TFP caused by adding CaM, which would give guite useful information as to the hydrophobic interaction between TFP and CaM. We

† From the Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Katahira, Sendai 980, Japan. *Received April 17, 1984.* This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Nissan Science Foundation to M.H.

[1] Abbreviations: CaM, calmodulin; TFP, trifluoperazine; K_d, dissociation constant; CD, circular dichroism; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

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describe in this paper that TFP gives rise to a positive CD band when Ca^{2+}-free CaM is added to the solution and that the positive induced CD band of TFP was diminished by adding alkaline metal monovalent cations. In addition, it was found that bivalent metal cations such as Mn^{2+}, Cd^{2+}, or Zn^{2+} induce strong negative CD bands to TFP in CaM solutions in the same manner as Ca^{2+}. The presence of the alkaline monovalent cation markedly affected the intensity of the induced CD band and the dissociation constant of those bivalent metal cations. Lastly, phenothiazines other than TFP also gave rise to induced CD bands which are comparable to that of TFP, while local anesthetics gave rise to only a small induced CD band.

Experimental Procedures

CaM was prepared from porcine brain by a modification of the methods previously described (Yazawa et al., 1980; Gopalakrishna & Andersson, 1982). Briefly, our method included precipitation with trichloroacetic acid, heat treatment, and chromatography on phenyl-Sepharose. The purity of the protein was checked by SDS gel electrophoresis.

Reagents used were of the highest guaranteed grade and were used without further purification. Doubly distilled water was used throughout the experiments. By using Chelex-100 of Bio-Gel, a trace amount of Ca^{2+}, ~30 μM, cannot be eliminated from the aqueous solution. The trace amount of Ca^{2+} did not affect the CD spectral results at all as will be mentioned later. TFP as a dimaleate form was purchased from Wako Pharmaceutical Co. Chlorpromazine, fluphenazine, and propericiazine were kind gifts from Yoshitomi Pharmaceutical Co. and were obtained through courtesy of Prof. Y. Nozawa of Gifu University. The pH values were strictly kept at 7.0 ± 0.1 unless otherwise noted. Since pH values of the TFP or TFP–CaM solutions were often fairly changed by adding CaM or various reagents to the solutions, the pH was always strictly checked before and after the CD measurements. The pH value was often adjusted by 0.1 M NaOH or 0.1 M HCl before each CD measurement. Every CD spectrum was measured after more than 20 min of sample preparations to make sure the interaction equilibrated. Every sample was made to stand at room temperature for more than 12 h after the first CD measurement. The CD spectrum was again measured after 12–48 h for several times to determine time-dependent spectral changes. Before and after the CD measurements, we always measured absorption spectra under the same conditions to confirm that the sample solutions have no excess optical absorption band other than those of TFP itself. Absorption spectra of all sample solutions studied here were essentially identical.

To avoid bacterial growth during 48 h, 0.02% NaN_3 was added to the TFP–CaM solution in some cases. No effect of the addition of 0.02% NaN_3 on CD spectra was noted. To confirm that CaM is still in a native form after 48 h at room temperature, we checked the 300-MHz ^1H NMR spectra and activities of CaM. The ^1H NMR spectrum of Ca^{2+}-bound CaM in 0.2 M KCl solution did not change at all after the sample solution was kept at room temperature (20 °C) for 48 h. Activities of phosphodiesterase were measured in a freshly prepared CaM solution and in a CaM solution which was kept at room temperature for 48 h. Phosphodiesterase activities were monitored at 265-nm absorbance for solutions composed of CaM-free phosphodiesterase (Boehringer), alkaline phosphatase (Boehringer), adenosine deaminase (Boehringer), and adenosine cyclic 3′,5′-phosphate (Boehringer) solutions (Sharma & Wang, 1979). Phosphodiesterase activities caused by both CaM solutions were identical. Thus, it was shown

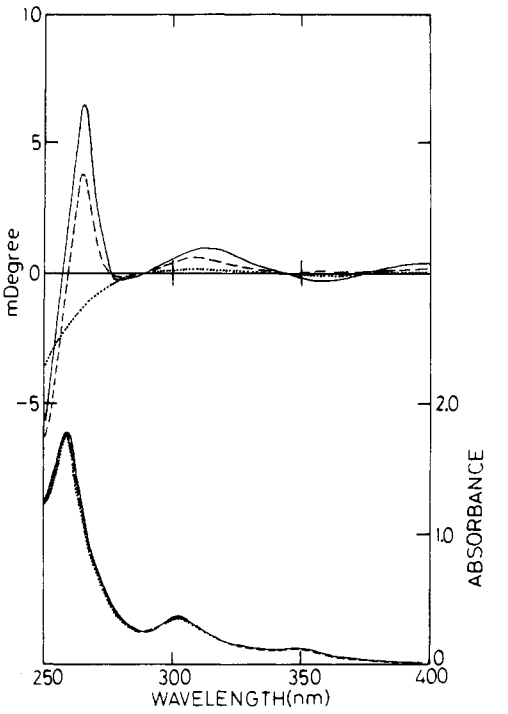

FIGURE 1: CD (upper) and absorption (lower) spectra of TFP (90 μM)–CaM (45 μM) solutions. The spectra were measured in distilled water at pH 5.3 (—) and pH 7.0 (- - -) and in 0.2 M KCl at pH 7.0 (···). The pH of the TFP–CaM solution in distilled water was 5.3, which was raised to 7.0 by adding a trace amount of a 0.1 M NaOH solution.

that CaM is still in a native state after being kept at room temperature for 48 h.

CD and absorption spectra were obtained at 20 °C on a JASCO J-500 spectropolarimeter equipped with a Data Processor DP-500 and a JASCO UVIDEC-510 spectrometer, respectively. CD spectra were obtained with the following parameters: path length, 1 cm (for the near-UV region) or 1 mm (for the Far-UV region); time constant, 16 s; scanning time, 5 nm/min; sensitivity, 1 mdeg (for the near-UV region) or 10 mdeg (for the far-UV region); spectral bandwidth, 2 nm. We described millidegree (mdeg) as the unit for the CD magnitude. By using the equation $[\theta] = HS/(10Cl)$ (where H is the CD intensity in centimeters, S is in millidegrees per centimeter, C is the molar concentration, and l is the path length in centimeters), one can easily convert the millidegree unit into the molar ellipticity, $[\theta]$ (in degrees centimeter squared per decimole). Thus, 1 mdeg in the text for the 90 μM TFP solution corresponds to $[\theta] = 1111$ deg·cm^2/dmol on the basis of the TFP concentration. The $[\theta]$ value can be converted into the $\Delta\epsilon$ value ($M^{-1}\cdot cm^{-1}$) (the difference in the values for the molar extinction coefficient between left and right circularly polarized light) by using $[\theta] = 3300\Delta\epsilon$ ($\Delta\epsilon = \epsilon_l - \epsilon_r$).

Results

Effects of Alkaline Metal Monovalent Cations on the Positive CD. Figure 1 shows CD spectra of the TFP (90 μM)–CaM (45 μM) solution in distilled water in the absence and presence of 0.2 M KCl. For the solution in the absence of KCl, a sharp CD band having a magnitude of 7 mdeg was observed on the positive side around 265 nm which corresponded to the absorption peak (long-axis polarized transition) of TFP. This peak was essentially not changed by adding up to 0.5 mM EGTA but was decreased by adding more than 0.5 mM EGTA. The positive peak around 265 nm was diminished by adding KCl as seen in Figure 1. Neither of the negative

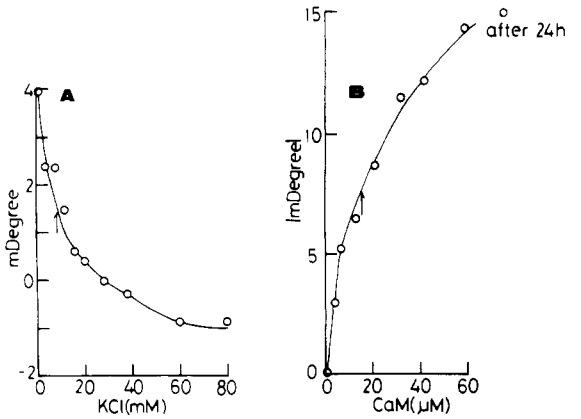

FIGURE 2: CD spectral titrations by KCl and CaM. (A) CD spectral changes around 265 nm of the TFP (90 μM)–CaM (45 μM) complex in distilled water caused by adding KCl. (B) CD spectral changes around 260 nm in absolute magnitude of TFP (90 μM) in 0.2 M KCl–4 mM $CaCl_2$ caused by adding CaM at pH 7.0.

Table I: Induced CD Spectra of TFP–CaM Solutions

metal cations	K_d bivalent cations[a]	K_d monovalent cations	CD magnitude[b]
Li^+–H_2O		10 mM	
Na^+–H_2O		10 mM	
K^+–H_2O		10 mM	
Rb^+–H_2O		10 mM	
Cs^+–H_2O		10 mM	
Ca^{2+}–0.2 M KCl	<50 μM		15
Ca^{2+}–0.2 M LiCl	<50 μM		11
Ca^{2+}–0.2 M NaCl	<50 μM		9
Ca^{2+}–0.2 M RbCl	<50 μM		13
Ca^{2+}–0.2 M CsCl	<50 μM		13
Ca^{2+}–H_2O	<50 μM		10
Mn^{2+}–0.2 M KCl	50 μM		13
Cd^{2+}–0.2 M KCl	40 μM		13
Zn^{2+}–0.2 M KCl	2 mM		12
Co^{2+}–0.2 M KCl	1 mM		6
Mn^{2+}–H_2O	20 μM		18
Cd^{2+}–H_2O	90 μM		17
Zn^{2+}–H_2O	0.5 mM		16
Co^{2+}–H_2O	1 mM		12
Mg^{2+}–0.2 M KCl	1 mM		5
Sr^{2+}–0.2 M KCl	0.2 mM		5
Ba^{2+}–0.2 M KCl	20 mM		2
Mg^{2+}–H_2O	1 mM		7
Sr^{2+}–H_2O	80 μM		15
Ba^{2+}–H_2O	20 mM		10

[a] Maximum K_d values were estimated from the induction of the CD band around 260 nm for TFP (90 μM)–CaM (45 μM) solutions as described in the text. [b] CD magnitudes were expressed in millidegrees for the above-mentioned solutions with a 1-cm cell at a sensitivity of 1 mdeg.

bands of TFP was observed in the presence of KCl. Since the pH value of the TFP–CaM solution in distilled water was nearly 5.3, the pH had to be raised to 7.0 to study the TFP–CaM interaction under physiological conditions by adding NaOH or KOH solutions. By doing so, the positive CD band around 265 nm was decreased by nearly 30% (Figure 1). The decrease of the positive CD band was observed at pH 7.0 by adding KCl, NaCl, LiCl, RbCl, or CsCl as well. The decrease of the CD band by adding KCl or other alkaline salts was monophasic (Figure 2A). The K_d value estimated from the decrease of the positive CD band was nearly 10 mM for the all alkaline monovalent metal cations (Table I). A marked difference of the K_d values among these alkaline metal cations was not noted. A pH variation experiment was not performed for the nonsalted TFP–CaM solution to avoid the confusion

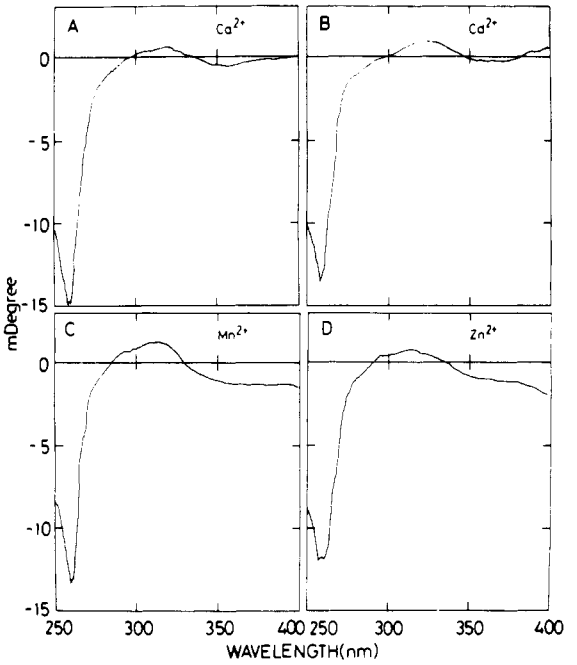

FIGURE 3: CD spectra of TFP–CaM in the presence of transition-metal cations. CD spectra of TFP (90 μM)–CaM (45 μM) in 0.2 M KCl solutions (pH 7.0) in the presence of 4 mM $CaCl_2$ (A), 0.8 mM $CdCl_2$ (B), 2 mM $MnCl_2$ (C), and 50 mM $ZnCl_2$ (D).

coming from the pH effect and the effect of alkaline metal addition.

Effects of $CaCl_2$ on the Positive CD. By addition of $CaCl_2$ to the TFP (90 μM)–CaM (45 μM) solution containing 0.2 M KCl, a fairly large negative CD band having a magnitude of 10 mdeg appeared around 260 nm corresponding to the absorption peak (long-axis polarized band) of TFP instead of the positive CD band. This negative CD band around 260 nm was time-dependently enlarged to 15 mdeg (Figure 3A) after 48 h as described previously (Shimizu & Hatano, 1983). Figure 3A is the spectrum essentially the same as that described by Shimizu & Hatano (1983). Here, base-line correction was done with the data processor. The induction of the negative CD band caused by adding $CaCl_2$ was also seen in 0.2 M LiCl, NaCl, RbCl, and CsCl solutions. Maximum K_d values for Ca^{2+} estimated from the induction of the negative CD bands caused by adding $CaCl_2$ were nearly 50 μM (Table I). No marked difference of K_d values for Ca^{2+} among the alkaline cation solutions was observed. The CD magnitudes were 11, 9, 13, and 13 mdeg for the TFP–CaM complex in the LiCl, NaCl, RbCl, and CsCl solutions, respectively (Table I).

Figure 2B shows a titration curve which was observed by adding CaM to a TFP (45 μM) solution consisting of 0.2 M KCl and 4 mM $CaCl_2$ at pH 7.0. The maximum K_d value for CaM from TFP was estimated to be nearly 15 μM. The CD magnitude reached nearly 15 mdeg by adding a sufficient amount of CaM in 5 h. This CD magnitude increased slightly after 24–48 h. This result is in contrast with that obtained by adding Ca^{2+} to the TFP–CaM solution in that the induced CD band caused by adding Ca^{2+} to the TFP–CaM solution containing 0.2 M KCl was time-dependently enlarged for more than 20 h as mentioned previously.

Effects of pH on the Negative CD. The pH effect of the CD spectrum of the TFP (90 μM)–CaM (45 μM) complex in a 0.2 M KCl–4 mM $CaCl_2$ solution was studied (Figure 4). The inset in Figure 4 shows that raising the pH to 11.8 resulted in a decrease in the intensity of the CD band at 260 nm as well as a shift in the wavelength from 260 to 264 nm.

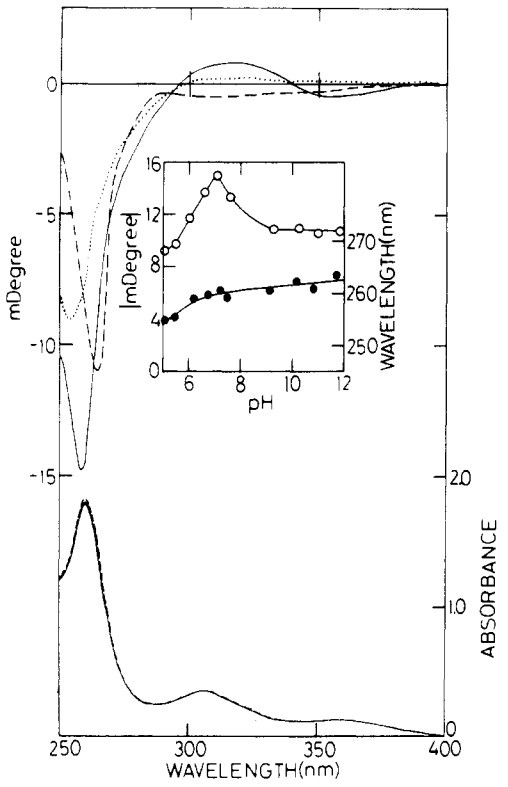

FIGURE 4: Effects of pH on the CD (upper) and absorption (lower) spectra of TFP–CaM solutions. CD spectra of TFP (90 μM)–CaM (45 μM) in 0.2 M KCl at pH 5.0 (···), 7.0 (—), and 11.8 (---). The inset shows changes in the position (●) and magnitude (○) of the TFP–CaM solution plotted against pH.

Lowering the pH resulted in a decrease in the intensity of the CD band, too, but this decrease was more marked than that observed by raising the pH. The apparent pK_a estimated from the decrease of the induced CD band was nearly 6.4.

Effect of Transition Metal Bivalent Cations on the Negative CD. The induction of the negative CD band was also observed by adding $MnCl_2$, $CdCl_2$, and $ZnCl_2$ to the TFP (90 μM)–CaM (45 μM) solution containing 0.2 M KCl (Figures 3B,C). K_d values estimated from the induction of the CD band were less than 50 μM, 40 μM, and 2 mM for Mn^{2+}, Cd^{2+}, and Zn^{2+}, respectively (Table I). The CD magnitudes of the TFP–CaM solution containing 0.2 M KCl were 13, 13, and 12 mdeg in the presence of 2 mM $MnCl_2$, 2 mM $CdCl_2$, and 50 mM $ZnCl_2$, respectively (Table I). Addition of $CoCl_2$ to the TFP–CaM solution containing 0.2 M KCl induced a small CD band around 260 nm having a magnitude of 6 mdeg. Addition of 0.1 mM $CuCl_2$ to the TFP–CaM solution containing 0.2 M KCl induced a negative CD band around 260 nm having a magnitude of 5 mdeg, but further addition of $CuCl_2$ interfered with the CD measurements due to the absorption of copper(II) complex. Measurements of the CD bands of the TFP–CaM solutions caused by adding $FeCl_2$, $FeCl_3$, adn $NiCl_2$ were not feasible due to the very strong absorption bands around 260 nm of $FeCl_2$, $FeCl_3$, and $NiCl_2$ per se. By addition of $TbCl_3$ to the TFP–CaM solution, heavy precipitates were formed, which made it impossible to obtain the CD spectrum. Practically no time-dependent increase in the CD magnitude was observed for transition-metal–TFP–CaM solutions.

Effects of Alkaline Earth Metal Bivalent Cations on the Negative CD. CD spectral changes caused by adding alkaline earth bivalent cations other than Ca^{2+} to the TFP (90 μM)–CaM (45 μM) solution containing 0.2 M KCl were studied. Additions of $MgCl_2$, $SrCl_2$, and $BaCl_2$ to the solution caused only small negative CD bands around 260 nm. The

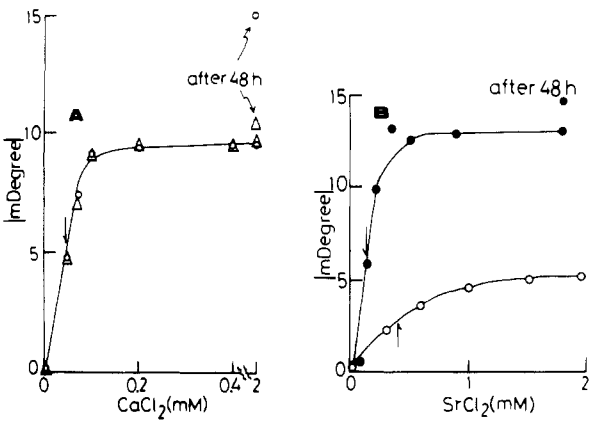

FIGURE 5: CD spectral titrations by $CaCl_2$ and $SrCl_2$. (A) CD spectral changes in absolute CD magnitude around 260 nm for TFP (90 μM)–CaM (45 μM) caused by adding $CaCl_2$ at pH 7.0 in distilled water (△) and in 0.2 M KCl (○). CD spectral magnitudes after 48 h of sample preparation are also shown in the right end of the upper region. (B) CD spectral changes in absolute CD magnitude around 260 nm for TFP (90 μM)–CaM (45 μM) at pH 7.0 caused by adding $SrCl_2$ in 0.2 M KCl (○) and in distilled water (●).

CD magnitudes were 5, 5, and <2 mdeg for the $MgCl_2$, $SrCl_2$, and $BaCl_2$ solutions, respectively (Table I). K_d values estimated from the induction of the negative CD bands were less than 1, 0.2, and 20 mM for Mg^{2+}, Sr^{2+}, and Ba^{2+}, respectively (Table I). Adding up to 5 mM $CaCl_2$ to the TFP–CaM solutions containing 2–4 mM alkaline earth cations did not make the small negative CD band larger at all. Addition of more than 1 mM $BeCl_2$ to the TFP–CaM solution formed heavy precipitates, which interfered with the detection of the CD band.

Effects of KCl on the Negative CD. Induced CD spectral features in distilled water were remarkably different from those in 0.2 M KCl solution (Figure 5, Table I). Thus, the time-dependent increase of the induced CD band of the TFP–CaM solution caused by adding $CaCl_2$ was observed only for the KCl solution and was not observed for the non-KCl solution (Figure 5A). The CD magnitude of the TFP–CaM solution containing only 2 mM $CaCl_2$ was much smaller than that in the 2 mM $CaCl_2$–0.2 M KCl solution (Table I). KCl did not affect the maximum K_d value for Ca^{2+} from the TFP–CaM complex as shown in Figure 5A.

In contrast to the Ca^{2+} solution, the CD magnitudes of the TFP–CaM complex in distilled water in the presence of $MnCl_2$, $CdCl_2$, $ZnCl_2$, $CoCl_2$, $SrCl_2$, or $BaCl_2$ were markedly larger than those in 0.2 M KCl solution (Table I). It was noted especially that the CD magnitudes of the TFP–CaM complex in the presence of $CoCl_2$, $SrCl_2$, and $BaCl_2$ in distilled water were 2 or 3 times as large as those in the 0.2 M KCl solution. The maximum K_d values obtained in distilled water for Mn^{2+}, Zn^{2+}, and Sr^{2+} were decidedly lower than those obtained in 0.2 M KCl (Table I). Figure 5B shows the effect of KCl on the induction of the CD band caused by adding $SrCl_2$ to the TFP–CaM solution as a typical case. No time-dependent change of the CD magnitude was observed for the TFP–CaM solution in distilled water.

Far-UV Region. CD spectra of the far-UV region (190–250 nm) of CaM were studied to know whether or not the backbone structure of CaM is changed by adding excess TFP. Ca^{2+}-free CaM had a negative CD around 220 nm with a magnitude of $[\theta]_{220nm}$ = 15000. The CD magnitude of Ca^{2+}-free CaM was increased to $[\theta]_{220nm}$ = 18000 by adding 2 mM Ca^{2+}. These CD spectral features of porcine CaM in the absence of TFP were the same as those reported for bovine CaM (Liu & Cheung, 1976; Wolff et al., 1977; Klee, 1977;

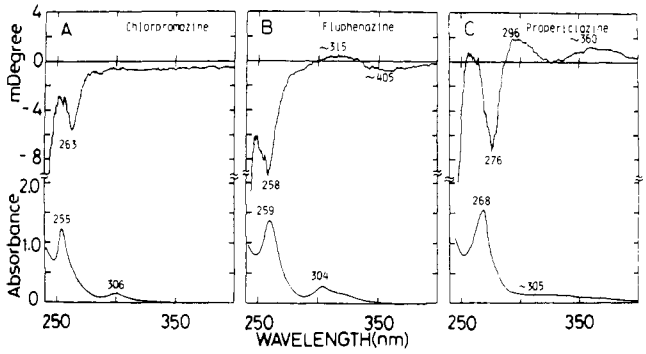

FIGURE 6: CD spectra of phenothiazine–CaM solutions. CD (upper) and absorption (lower) spectra of (A) chlorpromazine (4 μM)–CaM (12 μM), (B) fluphenazine (6 μM)–CaM (20 μM), and (C) propericiazine (4.5 μM)–CaM (34 μM) in 0.2 M KCl–4 mM $CaCl_2$ solutions (pH 7.0). Adding more CaM to the solutions did not increase the CD magnitude of these phenothiazine solutions.

Walsh & Stevens, 1978) in the absence of TFP. No marked change of the far-UV CD spectrum of CaM was observed by adding excess TFP in the presence of 0.2 M KCl and 4 mM $CaCl_2$. However, it should be emphasized that an increase of 25% in the CD magnitude around 220 nm was observed for Ca^{2+}-free CaM by adding excess TFP (not shown). Addition of 4 mM Ca^{2+} to the Ca^{2+}-free CaM–TFP solution did not change the negative CD band any further.

Interactions of Other Phenothiazines with CaM. Interactions of phenothiazine antipsychotic drugs, such as chlorpromazine, fluphenazine, and propericiazine, with CaM in the presence of 0.2 M KCl and 4 mM $CaCl_2$ were studied. As can be seen in Figure 6, these phenothiazine drugs decidedly gave rise to CD bands at the wavelengths corresponding to the absorption peaks of these compounds. The CD magnitudes of these drug–CaM complexes were smaller than that of the TFP–CaM complex. Local anesthetics, such as procaine, tetracaine, and dibucaine, gave rise to only small induced CD bands (not shown) corresponding to the absorption peaks of these drugs in the presence of CaM (45–90 μM), 0.2 M KCl, and 4 mM $CaCl_2$.

Finally, it should be noted that changes of the electronic absorption band of TFP caused by adding CaM in the presence or absence of $CaCl_2$, KCl, or other monovalent or bivalent metal cations were practically negligible (lower parts in Figures 1 and 4) in comparison with the CD spectral change during the course of our CD experiments. In addition, the TFP solution in the presence of adequate amounts of other proteins such as bovine serum albumin, concanavalin A, and thermolysin did not cause any induced CD band of TFP at all. Thus, the induced CD band of TFP is a very specific case observed only for the CaM solution.

Discussion

Induced CD is the phenomenon in which the CD band is induced at the absorption wavelength of the achiral molecule by the dissymmetric perturbation of the chiral species. For example, CD bands of symmetric organic aromatic compounds were induced by inclusion of these aromatic molecules into the cyclodextrin cavity and were used to determine the relative direction of the electronic transition dipole moment of the aromatic compounds (Shimizu, H., et al., 1982). When the achiral molecule is fixed relative to the bound chiral molecule, the CD signs depend on the polarization direction of transitions of the given achiral molecule. This is valid for the TFP molecule bound to CaM in the absence and presence of Ca^{2+} and K^+ ions (Figures 1 and 3). This is the first paper describing the induced CD spectral features of the phenothiazine solution in detail. It was found that this method, induced CD spectroscopy, is highly useful for studying drug–protein interactions. We have chosen to work at a TFP:CaM ratio of 2:1 since it is known that CaM binds 2 mol of TFP and additional moles of TFP are bound to CaM in a nonspecific manner (Levine & Weiss, 1977; Bromstrom et al., 1982; Roufagalis, 1982; Shimizu & Hatano, 1982).

Positive CD Spectra of TFP–CaM Solutions. We have previously described that TFP actually weakly interacts with Ca^{2+}-free CaM in terms of ^{19}F NMR spectroscopy (Shimizu & Hatano, 1983). The positive induced CD band around 265 nm (long-axis polarized transition of TFP itself) of the TFP–CaM solution in the absence of Ca^{2+} confirmed our suggestion that TFP interacts with Ca^{2+}-free CaM. It was suggested from ^{19}F NMR spectra (Shimizu & Hatano, 1983) that TFP interacts with Ca^{2+}-free CaM in a nonspecific manner. However, it is suggested in this study that TFP binds to a specific site of Ca^{2+}-free CaM, which induces the positive CD band of TFP. Gariépy & Hodges (1983) studied the cyanogen bromide fragment of rabbit skeletal troponin C as a model compound for elucidating the interaction of TFP with CaM. They suggested from ^1H NMR and CD spectral studies that the model peptide for CaM does not necessitate Ca^{2+} for specific TFP binding to the model peptide. Our finding is consistent with their suggestion. It was also found in this paper that alkaline metal monocations such as K^+, Na^+, Li^+, Rb^+, or Cs^+ markedly influence the interaction between TFP and Ca^{2+}-free CaM. Thus, it is suggested that the conformation of the Ca^{2+}-free CaM molecule interacted with TFP in distilled water is markedly different from that in the alkaline monovalent cation solutions or that the binding site of TFP on Ca^{2+}-free CaM in distilled water is different from that in alkaline metal solution because of a conformational change of Ca^{2+}-free CaM caused by adding the alkaline monovalent cations (Crouch & Klee, 1980). In this respect, participation of a carboxyl amino acid group such as Asp or Glu in CaM to the TFP–CaM interaction is implied. Gariépy & Hodges (1983) have suggested by homology of rabbit skeletal troponin C that even in the absence of Ca^{2+} TFP will probably bind to the region 82–92 in CaM, which is composed of both hydrophobic and negatively charged amino acids. The region 82–92 in porcine CaM (Watterman et al., 1980) is composed of four Glu, two Arg, one Ile, one Ala, two Phe, and one Val. Our findings are thus in accordance with their suggestion. The effect of salts on the interaction of TFP with CaM may be ascribed to a Donnan-type effect (Hoppe et al., 1983). That is, CaM has acidic amino acids such as Asp and Glu, the number of which is more than 3 times that of basic amino acids (Watterman et al., 1980; Means et al., 1982). A large excess of alkaline metal monocations will neutralize the negatively charged groups in the CaM molecule. The changes of the net charge or the protein conformation of CaM caused by monocations may alter the characteristics of the specific interaction between TFP and CaM. Affinities of K^+ or Na^+ for amino acids or peptides are lower than those of Mg^{2+} or Ca^{2+} (Hughes, 1981). The high K_d values, 10 mM, of the alkaline cations (K^+, Na^+, etc.) from the TFP–CaM complex may be in part ascribed to those differences in affinities.

It is ruled out that one of the reasons for the positive CD band of the TFP–Ca^{2+}-free CaM solution is trace amounts of Ca^{2+} (10^{-6}–10^{-5} M) dissolved in distilled water, since adding up to 0.5 mM EGTA did not change the positive CD band at all. Since EGTA itself is not dissolved in neutral distilled water, NaOH or KOH must be added to the EGTA solution to dissolve EGTA. The decrease of the positive CD band by

adding more than 0.5 mM EGTA must be due to Na^+ in the EGTA solution.

Negative Induced CD Spectra of the TFP–CaM Solutions. The positive sign of the CD band of the TFP–CaM solution in the absence of K^+ and Ca^{2+} reversed to the negative sign by adding Ca^{2+} or other bivalent cations. It is suggested, therefore, that the mode of TFP binding to CaM in the absence of Ca^{2+} is different from that in the presence of Ca^{2+}. The time-dependent change of the induced CD band was not concerned with a change in the backbone structure of CaM since the CD band of the Ca^{2+}-bound CaM in the far-UV region changed very little by adding TFP in 0.2 M KCl solution. Since titration studies involving the addition of bivalent metal cations were performed within 6 h, the contribution of the time-dependent change to the determination of the K_d value was practically negligible. The K_d values described here for Ca^{2+} and other bivalent metal cations from CaM should be maximum values. Since it was unfeasible to decrease the TFP concentration under our experimental conditions, correct dissociation constants could not be obtained. In addition, the contribution of the far-UV CD of CaM to the CD magnitude around 260 nm (10–20%) interfered in part with obtaining correct K_d values.

A marked decrease of the induced CD of the TFP–CaM complex in 0.2 M KCl–2.2 mM solution was observed below pH 7.0. Since the dissociation of Ca^{2+} from the TFP–CaM complex takes place at pH 5.4 in terms ^{43}Ca NMR (Shimizu, T., et al., 1982), it seems unlikely that the decrease of the negative induced CD band below pH 7.0 is directly related to Ca^{2+} dissociation. Rather, it seems likely that the CD change below pH 7.0 may be associated with carboxylate or imidazole groups of CaM. According to the suggestion by Gariépy & Hodges (1983), carboxylate groups of CaM are located in the proximity of TFP even in the presence of Ca^{2+}. Since His is at the 107-position in porcine CaM as in bovine CaM (Watterman et al., 1980), only carboxylate groups in CaM may still contribute to the negative induced CD band of the Ca^{2+}-bound CaM–TFP solution as for the Ca^{2+}-free CaM–TFP solution. Therefore, Ca^{2+} may change the conformation of Ca^{2+}-free CaM, leading to a change in the specific Ca^{2+}-free CaM–TFP interaction and resulting in the induction of the negative CD band. It was suggested from equilibrium dialysis studies that TFP partly dissociates from CaM above pH 7.5 (Levine & Weiss, 1977). The magnitude of the induced CD band of TFP–CaM was decreased by raising the pH to more than 7.5 (Figure 4). The decrease of the induced CD band in the alkaline region was smaller than that observed in the acidic region. Thus, the induced CD of TFP–CaM in the alkaline region may come from the interaction of a TFP molecule tightly bound to CaM of the two TFP molecules bound to CaM.

It has been reported that Mg^{2+}, Mn^{2+}, or Cd^{2+} can bind at a Ca^{2+} binding site of CaM and cause various effects on the conformation of CaM or the functions of CaM (Liu & Cheung, 1976; Klee, 1977; Wolff et al., 1977; Dedman et al., 1977; Walsh & Stevens, 1978; Crouch & Klee, 1980; Haiech et al., 1981; Shimizu, T., et al., 1982; Andersson et al., 1982, 1983; Cox & Harrison, 1983). We have found in this study that Mn^{2+}, Cd^{2+}, and Zn^{2+} do induce a large negative CD band in the TFP–CaM solution containing 0.2 M KCl. ^{113}Cd NMR studies (Andersson et al., 1982, 1983; Thulin et al., 1984) suggested that Cd^{2+} can bind at the Ca^{2+} binding sites in CaM. ^{113}Cd NMR also indicated that Zn^{2+} can bind at a high-affinity site for Cd^{2+} (Andersson et al., 1982). The change in the far-UV spectrum of CaM caused by adding Mn^{2+} was very close to that caused by adding Ca^{2+} in low ionic strength buffer (Wolff et al., 1977). Our results obtained for Mn^{2+}, Cd^{2+}, and Zn^{2+} from induced CD spectra are quite consistent with those obtained from other spectroscopic methods. Furthermore, it was found in our study that Mg^{2+} does not induce a large CD band for the CaM–TFP solution. From ^1H NMR studies (Seamon, 1980) and CD studies in the far-UV region (Dedman et al., 1977; Wolff et al., 1977), it was suggested that Mg^{2+} binding at the Ca^{2+} binding sites of CaM does not induce as large a change in conformation as Ca^{2+} (or Mn^{2+}) does in both high and low ionic strength buffers. Our induced CD results for Mg^{2+} are in accordance with these ^1H NMR and far-UV CD findings. Binding constants of transition-metal bivalent cations of carboxylate groups are much higher than those of alkaline earth bivalent cations by more than 1 order (Hughes, 1981). From equilibrium dialysis studies, it was suggested that K_d values of Mn^{2+} and Mg^{2+} from CaM are $\sim 10^{-6}$ and 10^{-4}–10^{-5} M, respectively (Wolff et al., 1977). Thus, the difference in the CD magnitudes between Mn^{2+} and Mg^{2+} may in part be ascribed to the difference in the affinities of these cations for CaM.

The effects of those bivalent metal cations on the induced CD spectra were very different between those seen in 0.2 M KCl solution and in distilled water. Here again, KCl markedly influences the interaction mode of TFP with CaM. It is known that the binding constants of metal cations to CaM in low ionic strength buffer are considerably different from those in high ionic strength buffer (Wolff et al., 1977; Dedman et al., 1977). This phenomenon may probably be due to competitive binding of alkaline monovalent cations with other bivalent cations (Haiech et al., 1981). Since it seems likely that negatively charged groups such as carboxylate groups of Glu in CaM interact with TFP (Gariépy & Hodges, 1983), K^+ may in part change the characteristics of the Ca^{2+}-bound CaM–TFP interaction as observed for the Ca^{2+}-free CaM–TFP solution. The Donnan-type effect (Hoppe et al., 1983) in aqueous solution in the absence of adequate salts is again suggested for the interpretation of the CD results.

It may be controversial whether bivalent metal cations are bound to the high-affinity or low-affinity site of CaM. It is difficult to conclude the problem at this point. However, it seems most likely that (1) bivalent metal cations which induce the negative CD band of TFP are bound to the same specific site(s) in CaM and (2) bivalent metal cations which are bound to the high-affinity site for Ca^{2+} in CaM will cause the negative CD band of TFP. The reasons for the latter suggestion are that (1) maximum K_d values for bivalent cations from CaM are fairly low and (2) KCl seems to inhibit the binding of the bivalent cations to CaM (Table I) except for Ca^{2+}. It is suggested from quadrupolar metal NMR studies (our unpublished results) that KCl markedly affects the interactions of Mg^{2+} and Zn^{2+}, probably bound at the high-affinity site, for Ca^{2+} in CaM. As an exceptional case, the presence of KCl seems to promote Ca^{2+} binding to CaM from induced CD spectra. However, it seems most likely that KCl promotes the hydrophobic interaction of TFP with Ca^{2+}–CaM rather than promoting Ca^{2+} binding to CaM, because the affinity of Ca^{2+} for CaM is partly inhibited by 0.1 M KCl (Wolff et al., 1977; Dedman et al., 1977). In other words, negative induced CD spectra of the TFP–CaM complex may be influenced by both the affinity of the bivalent metal cations for CaM and the hydrophobic interaction between TFP and CaM. The absence of KCl in the TFP–CaM solution may increase the affinities of the bivalent metal cations (except for Ca^{2+}) for CaM, since K^+ may compete with bivalent metal ions to bind at negatively

charged groups of CaM. On the other hand, the presence of KCl in TFP–CaM solutions may increase the hydrophobic interaction between TFP and CaM, which will be more effective than the decrease of Ca^{2+} affinity for CaM by the presence of KCl. This may be the reason for the activation off CaM specifically by Ca^{2+} and for nonactivation of CaM by Mg^{2+}, Zn^{2+}, or other bivalent metal cations under the physiological conditions of high ionic strength.

It was found that the binding fashion of the phenothiazines to CaM is different from that of the local anesthetics in terms of the induced CD spectra. The binding constants of the phenothiazines to CaM are much higher than those of the local anesthetics to CaM by more than 1 order (Roufogalis, 1982). The low binding abilities of the local anesthetics to CaM may be attributed to the small induced CD bands of the drug–CaM solutions. The low hydrophobic character of the local anesthetics compared with that of the phenothiazines may be part of the reason for the weak local anesthetic–CaM interactions.

Far-UV Region. The CD spectrum in the far-UV region of Ca^{2+}-bound CaM was hardly changed by adding excess TFP, but that of Ca^{2+}-free CaM was changed by adding excess TFP. The same CD spectral findings were observed for the model peptide of CaM, a cyanogen bromide fragment of troponin C (Gariépy & Hodges, 1983). Namely, the negative CD band around 220 nm of the Ca^{2+}-free peptide was intensified by adding Ca^{2+}, as observed for CaM. TFP addition to the Ca^{2+}-free peptide also increased the negative CD band of the peptide by ~30%. In addition, adding excess Ca^{2+} to the Ca^{2+}-free peptide-TFP solution intensified the CD band further by ~25%. Thus, the effect of Ca^{2+} on the Ca^{2+}-free peptide is not the same as that of TFP. For CaM, however, the increased CD band of the Ca^{2+}-free CaM-TFP solution was not intensified further by adding excess Ca^{2+}. Thus, it seems likely that the effect of TFP on the negative CD band of Ca^{2+}-free CaM is the same as that of Ca^{2+}. Even so, the structural similarity of CaM and the cyanogen bromide fragment of troponin C is implied here as has been suggested by Gariépy & Hodges (1983). It was found in our study that CaM does not necessitate the Ca^{2+} binding to cause a conformation of proper geometry for TFP binding as has been suggested for the model peptide (Gariépy & Hodges, 1983). In addition, the backbone structure of Ca^{2+}-bound CaM, which has more α-helix compared with Ca^{2+}-free CaM, may be induced by binding of TFP to Ca^{2+}-free CaM.

We have tried in this paper to obtain useful diagnostic knowledge regarding the interaction of TFP with CaM. The results obtained may serve as a useful working hypothesis in further pursuits of interactions of other antagonists with CaM.

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Registry No. TFP, 117-89-5; Li, 7439-93-2; Na, 7440-23-5; K, 7440-09-7; Rb, 7440-17-7; Cs, 7440-46-2; Ca, 7440-70-2; Mn, 7439-96-5; Cd, 7440-43-9; Zn, 7440-66-6; Co, 7440-48-4; Mg, 7439-95-4; Sr, 7440-24-6; Ba, 7440-39-3; chlorpromazine, 50-53-3; fluphenazine, 69-23-8; propericiazine, 2622-26-6.

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