membrane proteins as well as the formation of phase-separated domains will undoubtedly affect the efficiency and kinetic parameters of membrane-located biochemical reactions.

Registry No. Cytochrome b_5, 9035-39-6; DMPC, 13699-48-4; DPPC, 2644-64-6.

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Chlortetracycline as a Probe of Membrane-Associated Calcium and Magnesium: Interaction with Red Cell Membranes, Phospholipids, and Proteins Monitored by Fluorescence and Circular Dichroism†

Allan S. Schneider,* Ruth Herz, and Martin Sonenberg

ABSTRACT: The fluorescence emission and circular dichroism spectra of chlortetracycline (CTC) have been measured, including the effects of multivalent cations (Ca, Mg, La), of medium polarity, and of interaction with human red cell membranes, lipids, and a variety of proteins. An obligatory role of Ca in the association of CTC with membranes was demonstrated. Binding and kinetic constants for the CTC–Ca chelate interaction with membranes and phospholipids were determined. The results suggest that the CTC–Ca chelate fluorescence is greatly enhanced in the vicinity of membrane phospholipid head groups. The circular dichroism spectra indicate a number of distinct CTC conformations corresponding to chelation of specific cations, to interaction with membranes and phospholipids, and to medium polarity. The high quantum yield CTC–Ca conformation associated with membranes or phospholipids was identified by its characteristic circular dichroism spectrum and is different from the CTC–Ca conformation in nonpolar media (80% methanol).

Membrane calcium translocation is involved in many aspects of cell physiology including cellular secretion, muscle contraction, mitochondrial function, and membrane transduction of hormonal messages. The use of tetracyclines as a fluorescent probe of membrane-associated calcium and magnesium was first proposed by Caswell and Hutchison in 1971, and there have been many applications to a variety of functioning membrane systems since then. We have previously used chlortetracycline (CTC)[1] fluorescence to probe membrane

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[1] Abbreviations: CTC, chlortetracycline; RCM, red cell membrane(s); CD, circular dichroism; LL, lysolecithin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Ca translocation in squid and lobster axons during the action potential on a millisecond time scale (Hallett et al., 1972). We have also shown that CTC fluorescence is exquisitely sensitive to membrane-associated Ca by demonstrating 2 order of magnitude quantum yield enhancement relative to CTC alone (Hallett et al., 1972; Schneider et al., 1978). CTC fluorescence has also been applied to studies of Ca and Mg movements in mitochondria (DuBuy & Showacre, 1961; Caswell, 1972; Luthra & Olson, 1976, 1978; Babcock et al., 1979), sarcoplasmic reticulum (Caswell & Warren, 1972; Nagasaki & Kasai, 1980), exocrine pancreatic acinar cells (Chandler & Williams, 1977, 1978a,b), endocrine pancreatic islets (Taljedal, 1974, 1978; Gagerman et al., 1980), brain synapotosomes (Schaffer & Olson, 1976; Carvalho, 1978), leukocytes (Takeshige et al., 1980), smooth muscle membranes (Uchida, 1980), platelets (Feinstein, 1980), and erythrocytes (Behn et al., 1977).

Although tetracyclines have been widely applied as fluorescent probes of membrane-associated divalent cations, there remain important unanswered questions about the interactions of the Ca- and Mg–CTC chelates with membranes. Thus, the identity of the membrane compartments monitored by the CTC–chelate fluorescence, the amount and kinetics of chelate association with membranes, the spectroscopic parameters distinguishing between Ca- and Mg–CTC–membrane complexes, and the various conformational states of the CTC molecule induced by chelation of specific cations and interaction with membranes remain unknown. In the present paper, we investigate the effects of erythrocyte membranes, phospholipids, and a diversity of proteins on the spectroscopic properties of the CTC–Ca and –Mg chelates in an effort to resolve some of these questions.

Materials and Methods

Chlortetracycline hydrochloride was obtained from Nutritional Biochemical Corp. and was used without further purification. CTC is unstable in alkaline media, and fresh solutions were made daily immediately prior to making spectral measurements. Lysolecithin and cholesterol were obtained from Nutritional Biochemical Corp. Both synthetic and natural lysolecithins were employed in experiments, and no difference was found. The following proteins were obtained from Nutritional Biochemical Corp.: α-casein, β-lipoprotein (bovine Cohn fraction IV-4), bovine glycoprotein (fraction VI), ovalbumin, and fibrin. β-Lactoglobulin was obtained from Sigma Chemical Corp. Two additional protein fractions were isolated from purified red cell membranes by following the procedure of Rosenberg & Guidotti (1969) for their membrane fractions I and II. These represent a low ionic strength EDTA isolate (RCM fraction I) containing Spectrin and a high ionic strength (0.8 M NaCl) isolate (RCM fraction II).

Red cell membranes were prepared from fresh human blood by a procedure of gradual hypotonic hemolysis in progressively lower osmolarity Tris buffer, pH 7.4, solutions as previously described (Schneider et al., 1970). Final washes of the ghosts were in 25 mosM Tris-HCl, and 1 mM $CaCl_2$ was added to the medium to minimize ghost protein solubilization during storage overnight. The membranes were given a final wash immediately prior to preparation of samples for spectroscopic measurements.

Circular dichroism spectra were recorded by using a Cary 60 spectropolarimeter with a 6002 circular dichroism attachment in 1- or 5-mm cylindrical cuvettes. Fluorescence emission spectra were measured in a Hitachi Perkin-Elmer MPF 2a spectrofluorometer and a Cary 14 spectrophotometer modified for fluorescence detection. The CTC–calcium emission peak on these instruments was at 530 nm and was uncorrected for phototube spectral sensitivity. A corrected emission spectrum was obtained on a Shoeffel spectrofluorometer and gave an emission peak at 550 nm for the CTC–calcium chelate. Since most of the measurements reported here were made with the Perkin-Elmer instrument, we reported uncorrected fluorescence emission spectra directly observed. One-centimeter path fluorescence cuvettes were used for the emission spectra measurements. The excitation wavelength was set at 400 nm, and emission spectra were scanned from 400 to 700 nm. Unless otherwise indicated, all samples were in 10–20 mM Tris-HCl buffer, pH 7.3.

For measurement of association kinetics, the reaction was initiated in the fluorescence cuvette by adding CTC (50 μM) either to Tris buffer (pH 7.3) containing Ca (5 mM) or to Tris buffer containing Ca (5 mM) + RCM (0.1 mg of protein/mL), and the fluorescence was immediately recorded as a function of time. For measurement of dissociation kinetics, the Ca–CTC–RCM complex was pelleted, and an aliquot of pellet was diluted 1:30 in Tris buffer directly in a fluorescence cuvette. Recording of the decrease in fluorescence was begun immediately following dilution of the CTC–Ca–RCM complex. The time for mixing and initiating fluorescence measurements was under 5 s.

Results

In order to gain insight into the chemical nature of the membrane compartments monitored by the CTC–chelate fluorescence, we have compared the spectral properties of the CTC–cation associated with membranes with those for the CTC–cation associated with proteins and lipids. Figure 1 compares the effect of red cell membranes (RCM) and a variety of lipids and proteins on the fluorescence emission spectra of the CTC–Ca chelate. In Figure 1a, we note that while Ca enhances the emission about an order of magnitude relative to CTC alone, the effect of membranes without Ca is a much smaller enhancement of only about 2-fold. The addition of both membranes and Ca gives a fluorescent emission enhancement of almost 2 orders of magnitude above CTC alone, and an order of magnitude above the CTC–Ca chelate. In Figure 1b, we note that a similar fluorescence response of the CTC–Ca chelate is obtained with a phospholipid (lysolecithin) but not with cholesterol. Figure 1c shows that of the variety of proteins tried, including two RCM protein fractions, only α-casein, a phosphoprotein, gave an enhancement of fluorescence comparable to that for the intact membrane.

In Figure 2, we compare the effects of red cell membranes, a phospholipid (lysolecithin), and a phosphoprotein (α-casein) on the fluorescence emission of the CTC–Ca vs. the CTC–Mg chelates. Figure 2a shows that while the CTC–Mg fluorescence is greater than that of CTC–Ca, the membrane-induced enhancement of the CTC–Mg chelate fluorescence is considerably smaller than that for the CTC–Ca chelate. In addition, there is a 10-nm shift to shorter wavelengths for the Mg chelate relative to the Ca chelate (520 vs. 530 nm). The fluorescence response of the Ca- and Mg–CTC chelates to lysolecithin and α-casein (panels b and c, respectively, of Figure 2) showed similar relative enhancements and wavelength shifts to those induced by the membranes.

The sensitivity of the CTC fluorescence response to the divalent cation concentration in the presence and absence of membranes was measured (data not shown). The greatest sensitivity was found below 0.5 mM cation at a CTC concentration of 50 μM. The fluorescence response saturates

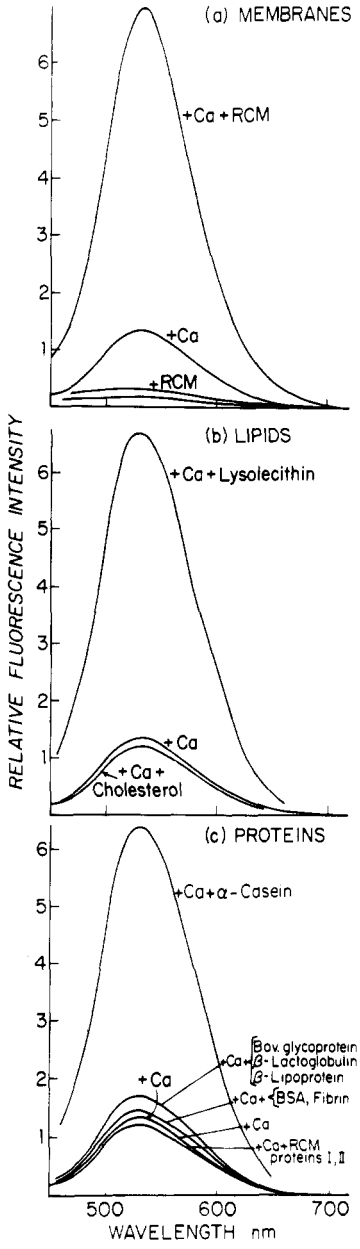

FIGURE 1: Fluorescence emission of chlortetracycline (CTC) as affected by calcium, membranes, lipids, and proteins. (a) CTC ± Ca ± red cell membranes (RCM); (b) CTC + Ca ± lysolecithin or cholesterol; (c) CTC + Ca ± proteins as indicated. Concentrations: CTC, 0.1 mM; Ca, 5 mM; RCM, 0.1 mg of membrane protein/mL; lipids and proteins, 0.1 mg/mL; Tris-HCl buffer, pH 7.3, 10 mM. The excitation wavelength was 400 nm.

Table I: Calcium Requirement for CTC Binding to Red Cell Membranes[a]

	supernatant absorbance		% membrane-bound CTC
	before RCM	after RCM	
CTC	0.55	0.54	0
CTC + Ca	0.77	0.42	45

[a] Samples were centrifuged at 30000*g* for 1 h prior to measurement of peak supernatant absorbance. Absorption peaks: CTC = 370 nm; CTC + Ca = 380 nm. Concentrations: CTC = 0.1 mM, Ca = 5 mM, RCM = 0.1 mg of membrane protein/mL.

above 0.5 mM divalent cation concentration at a CTC concentration of 50 μM.

The binding of CTC to red cell membranes in the presence and absence of Ca is shown in Table I. The absorbance of

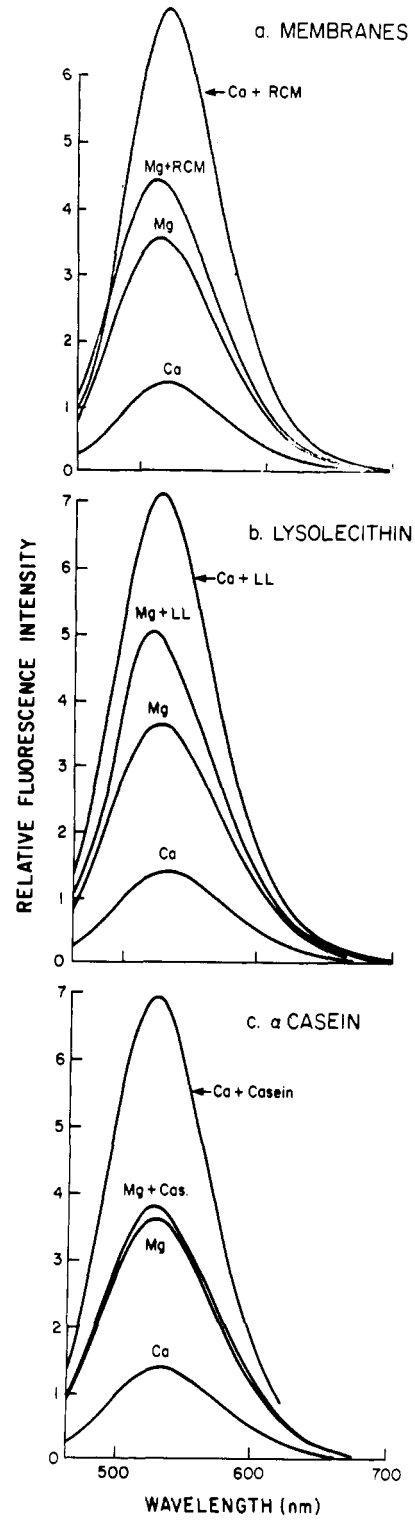

FIGURE 2: Fluorescence emission of Ca– vs. Mg–CTC chelates as affected by (a) red cell membranes (0.1 mg of membrane protein/mL), (b) lysolecithin (0.1 mg/mL), and (c) α-casein (0.1 mg/mL). [CTC] = 0.1 mM; [Ca] and [Mg] = 5 mM. The excitation wavelength was 400 nm.

CTC remaining in the supernatant after incubation with membranes is compared with the total CTC absorbance present before addition of membranes. The difference represents the amount pelleted with the membranes and presumably bound. In the absence of Ca, there is little or no binding of CTC to the membranes, whereas in the presence of Ca, about 45% of the CTC present is pelleted with the membranes and presumably bound. The volume of the

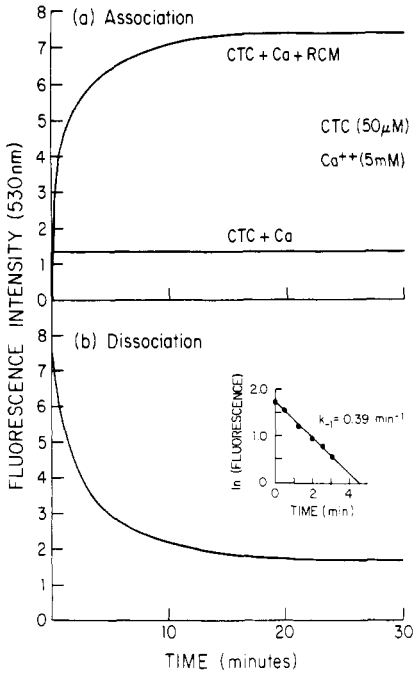

FIGURE 3: Kinetics of association (a) and dissociation (b) of CTC with Ca and red cell membranes. (a) The association reaction was initiated by adding CTC (50 μM) to Tris buffer, pH 7.3, containing either Ca (5 mM) or Ca (5 mM) plus membranes (0.1 mg of protein/mL) directly in the fluorescence cuvette. (b) Dissociation was initiated by diluting an aliquot of pelleted CTC–Ca–RCM complex with Tris buffer (1:30) directly in a fluorescence cuvette. The lag time between mixing sample components and recording the fluorescence emission was less than 5 s. The excitation wavelength was 400 nm. The inset in (b) is a plot of ln fluorescence vs. time whose slope is used for estimation of the dissociation rate constant k_{-1}.

membrane pellet is negligible compared to total volume present (3%) and could not account for removal of 45% of the total CTC by trapping inside the erythrocyte ghosts. If the reaction Ch + M ⇄ MCh is assumed to occur, where Ch is the CTC–Ca chelate, M is the red cell membrane, and MCh is the membrane–Ca–CTC complex, then it is possible to derive a binding dissociation constant by means of a double-reciprocal plot of 1/[MCh] vs. 1/[M]. The slope of such a plot yields a K_D of 1.3×10^{-4} mol of membrane phospholipid/L.

The kinetics of association and dissociation of CTC with Ca and of the CTC–Ca chelate with red cell membranes are shown in Figure 3. The fluorescence response is used as a measure of CTC association with Ca and CTC–Ca association with membranes. The association of CTC with Ca occurs more rapidly than the time required to initiate fluorescence measurements following manual mixing (5 s). The kinetics of the chelate interaction with the membrane are somewhat slower. The association of chelate with membrane is 50% complete in 1 min and 100% complete in about 15 min; 50% dissociation occurs in about 2 min. One can derive a dissociation rate constant, k_{-1}, for the assumed reaction

$$\mathrm{MCh} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \mathrm{Ch} + \mathrm{M}$$

from the data in Figure 3b by noting that the membrane–chelate concentration is proportional to the fluorescence. Since dissociation dominates the reversible reaction immediately following dilution of MCh in buffer, a plot of ln [MCh] (ln fluorescence) vs. time should be linear with a slope equal to k_{-1}. Such is found to be the case as seen in the inset in Figure 3b, and the resulting $k_{-1} = 0.39\ \mathrm{min}^{-1}$.

In Figure 4, we show the effect of lysolecithin concentration on the enhancement of CTC–Ca chelate fluorescence. There

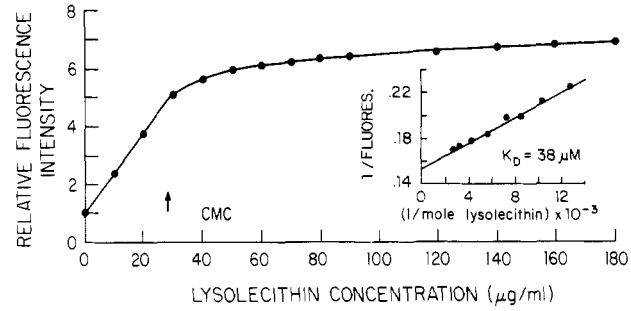

FIGURE 4: Effect of lysolecithin concentration on the CTC fluorescence emission at 530 nm (excitation wavelength 400 nm) in the presence of Ca. The arrow indicates the critical micelle concentration (cmc) estimated from Robinson & Saunders (1958). [CTC] = 50 μM; [Ca] = 5 mM. The inset shows a double-reciprocal plot with extrapolation to infinite lysolecithin concentration used for determination of the dissociation constant, K_D, for the dissociation of the Ca–CTC chelate from lysolecithin.

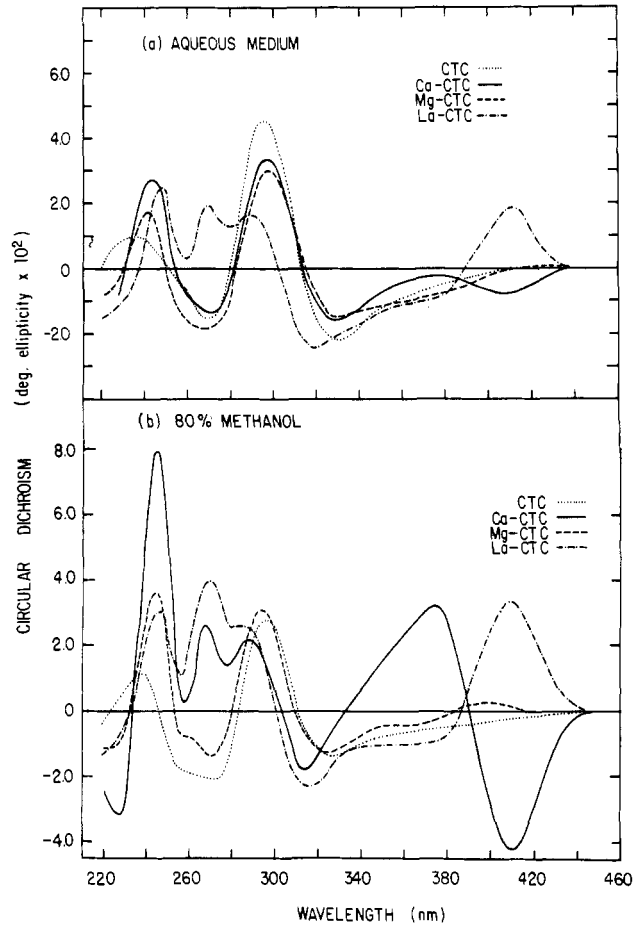

FIGURE 5: Circular dichroism spectra of CTC in the absence and presence of Ca, Mg, or La. (a) Aqueous medium, Tris-HCl, pH 7.0, 20 mM; (b) 80% methanol–20% aqueous media as in (a). [CTC] = 50 μM; [Ca], [Mg], and [La] = 1 mM.

is a leveling of the rise in fluorescence near the critical micelle concentration (cmc), and no sharp increase in fluorescence occurs as micelles begin forming. The dissociation constant for the CTC–Ca chelate interaction with lysolecithin, determined from a double-reciprocal plot (inset of Figure 4), is $K_D = 3.8 \times 10^{-5}$ M.

The circular dichroism spectra of CTC and its Ca, Mg, and La chelates are shown in Figure 5 in aqueous media (a) and in 80% methanol (b). There are two regions of the spectra that are sensitive to the particular multivalent cation chelated, to the polarity of the medium, and to association of the

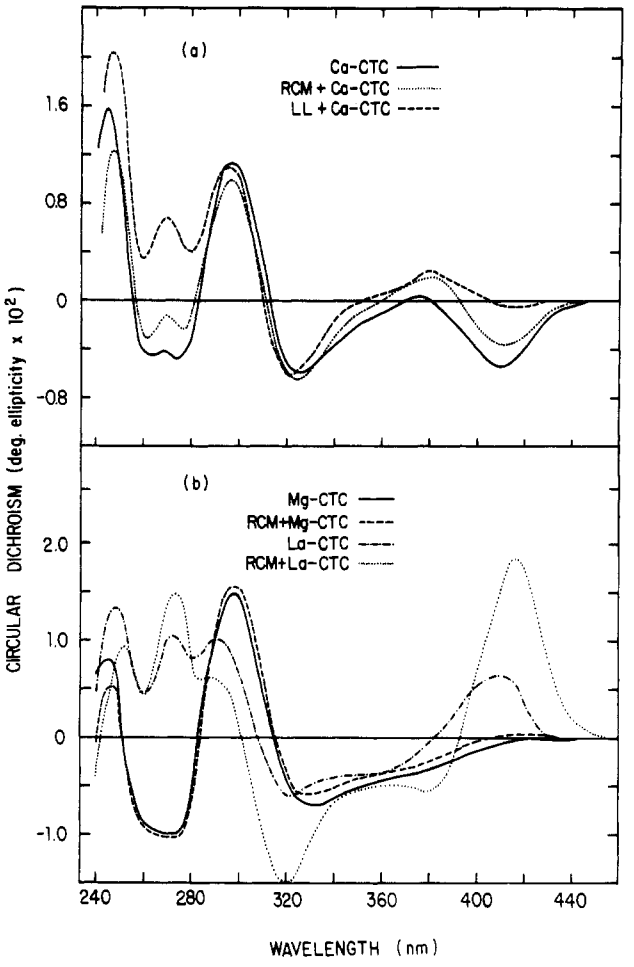

FIGURE 6: Effects of red cell membranes on the circular dichroism spectra of CTC–cation chelates. (a) RCM (0.1 mg/mL) and lysolecithin (0.1 mg/mL) effects on the CTC (50 μM)–Ca (5 mM) spectrum. (b) RCM (0.1 mg/mL) effects on the CTC (200 μM)–Mg (2 mM) and the CTC (200 μM)–La (0.5 mM) spectra. Both sets of spectra were in Tris-HCl buffer, pH 7.3.

CTC–Ca chelate with membranes and phospholipids. These two spectral regions lie in the vicinities of 400 and 270 nm. Figure 5 shows a characteristic negative 410-nm band for Ca–CTC which is greatly enhanced in 80% methanol. The La–CTC chelate shows a similar 410-nm band but of opposite sign, while the Mg–CTC chelate and the unchelated CTC have only very weak CD bands in this region. The other sensitive region of the CD spectrum is seen to be near 270 nm in Figure 5. The Ca and Mg chelates as well as unchelated CTC exhibit a negative band near 270 nm in aqueous media while the La chelate shows a positive band in this region (Figure 5a). Alteration of medium polarity with 80% methanol has a dramatic effect on the Ca–CTC CD bands, causing the 270-nm negative band to reverse sign.

Figure 6 shows the effects of red cell membranes on the CD spectra and conformation of various cation–CTC chelates. Interaction with membranes is seen to induce characteristic spectral changes for the Ca–CTC chelate in the same direction as interaction with lysolecithin (Figure 6a). Membranes enhance the CD bands of the La–CTC chelate while having little or no effect on those of the Mg–CTC chelate.

Discussion

The results reported above are relevant to a number of questions on the interaction of the CTC–Ca chelate with biological membranes. The 2 orders of magnitude fluorescence enhancement found for CTC plus Ca in the presence of membranes is also found in the presence of a phospholipid, lysolecithin, and a phosphoprotein, α-casein, but not other proteins, including two protein fractions derived from red cell membranes. Common to those systems inducing the characteristic large increase in CTC quantum yield is the presence of phosphate groups. This would suggest that CTC–Ca fluorescence signals in membranes could be monitoring the region of membrane phospholipid head groups. Further support for this location in the membrane for the CTC–Ca chelate comes from the observation that red cell membranes, phospholipids, and a phosphoprotein (α-casein) all produce the same relative effect on the Ca– vs. Mg–CTC fluorescence response (Figure 2). The circular dichroism spectra of Figure 6a also show similarities between the Ca–CTC conformation associated with membranes and phospholipids.

The question arises as to whether the Ca–CTC chelate is sensing the polar head group region of the membrane phospholipids or the nonpolar hydrocarbon membrane core. Nonpolar solvents are known to enhance the fluorescence of the CTC–Ca chelate and induce a special conformational state of the Ca chelate (Caswell & Hutchison, 1971a,b; Newman & Frank, 1976). Thus, it is conceivable that the Ca–CTC fluorescence signal derives from the nonpolar hydrocarbon core of the membrane. Two lines of evidence suggest that this is not the case. First, upon formation of lysolecithin micelles containing a hydrocarbon core, there is no sharp increase in the CTC–Ca fluorescence associated with the micelle beyond the enhancement that has occurred for the interaction of the chelate with solubilized lysolecithin monomers. Such an increase might be expected if the CTC–Ca fluorescence enhancement were associated with a partitioning into the nonpolar hydrocarbon core of the membrane. Second, there is a characteristic large enhancement of the negative CD band near 410 nm upon transfer of CTC–Ca from aqueous media to a less polar environment such as methanol (Figure 5b) or octanol. However, upon interaction of CTC–Ca with red cell membranes or lysolecithin, the opposite is found: the 410-nm CD trough is diminished (Figure 6a). The results shown in Figures 1, 2, 4, and 6 would thus be consistent with the CTC–Ca membrane fluorescence signal deriving, at least in part, from the polar head group region of membrane phospholipids.

The question of whether CTC–Ca has a special chelate binding site in the membrane or whether CTC fluorescence monitors native membrane Ca binding sites is at present not resolved. Both models would be sensitive to changes in Ca concentration in the membrane environment and may be operationally indistinguishable. Charged phospholipid head groups would be natural Ca binding sites, and the present data would be compatible with CTC fluorescence monitoring Ca in the vicinity of such membrane sites. CTC has been suggested to locate on the inner side of several membrane systems (Hallett et al., 1972; Caswell, 1972; Behn et al., 1977), and phospholipids carrying a net negative charge (e.g., phosphatidylserine) are also preferentially distributed on the inner half of the membrane bilyaer (Bretscher & Raff, 1975). Since Ca can interact with several negatively charged sites on the membrane, it is not unreasonable to assume that membrane-associated CTC fluorescence could originate in more than one region of the membrane.

The results shown in Table I and Figure 3 are relevant to the mode of binding and the kinetics of CTC association with biological membranes. In the absence of Ca, there is no significant CTC association with the red cell membrane. Ad-

dition of Ca causes about half of the CTC (0.1 mM) present in solution to bind to and pellet with the membranes (Table I). Thus, a divalent cation linkage may be required for CTC association with red cell membranes. However, the kinetics of CTC interaction with Ca are sufficiently rapid (Figure 3a) that the membrane would see mainly the CTC–Ca chelate under the conditions of the binding experiment. The chelate could either exchange its Ca for another Ca at a membrane Ca binding site or find a membrane site of its own to associate with. As pointed out above, both cases could yield fluorescence signals in response to changes in the local Ca concentration, as, for example, during membrane Ca translocation. The interaction of CTC (50 μM) with Ca (5 mM) is considerably more rapid, i.e., complete in less than 5 s, than the much slower kinetics for the CTC–Ca chelate interaction with red cell membranes (Figure 3a). This could conceivably be related to the chelate having to penetrate the membrane in order to associate with sites on the inner membrane surface or possibly be related to the kinetics of Ca binding or exchange between membrane and chelate.

Different conformational states of the Ca–, Mg–, and La–CTC chelates are readily apparent from their distinct CD spectra (Figure 5). The opposite signs of both the 270- and 410-nm bands for the aqueous Ca– and La–CTC chelates suggest structures of opposite chiral asymmetry. A characteristic Ca–CTC conformation in 80% methanol is indicated in the CD spectrum with a large double Cotton effect centered around 390 nM (410-nm negative band; 375-nm positive band) and a reversal in sign of the 270-nm band. The double Cotton effect can be generated by exciton interactions when close proximity occurs between like chromophores such as dimerized CTC molecules. Mitscher et al. (1970) have proposed such a structure in the form of an octahedral tetracycline metal chelate.

The conformation of the high quantum yield state of CTC associated with Ca and either membranes of phospholipids is reflected in its CD spectra (Figure 6a). If we assume that CTC chelation of a first Ca induces a sign reversal (negative to positive) of the band in the 260–270-nm region (Newman & Frank, 1976), then the phospholipids and membranes might stabilize a CTC conformation with only one chelated Ca. The reduction of the long-wavelength double Cotton effect (410-nm negative, 375-nm positive) by lysolecithin and membranes could similarly imply a CTC conformation with lower affinity of a second Ca binding site. Thus, the high quantum yield conformation of CTC associated with Ca and membranes could be a single Ca binding conformation with spectral properties different from those for a two Ca binding structure in nonpolar media (Figure 6a vs. Figure 5b). Recognizing that the spectra of Figure 6a are a composite of free CTC–Ca chelate and CTC–Ca associated with membranes, it is possible that the latter could resemble the La–CTC spectra with membrane-induced changes in chiral asymmetry.

To summarize the conformational studies, we have indicated a number of distinct CTC conformational states by characteristic CD spectra representing the following: (1) an unchelated CTC conformation (I) closely related to the Mg–CTC conformation with a negative 270-nm band and the absence of significant CD bands in the region of 400 nm; (2) a Ca–CTC conformation (II) associated with membranes or phospholipids having a positive 270-nm CD band and no significant bands in the 400-nm region; (3) a Ca–CTC conformation (III) in nonpolar media having a positive 270-nm band and a long-wavelength double Cotton effect with a negative 410-nm trough; and (4) a CTC conformation (IV) with chelated La ions having a positive 270-nm band and a long-wavelength double Cotton effect with a positive 410-nm band. Interpretation of CD spectra of CTC in aqueous media interacting with cations and membranes will be aided by an appreciation of the possibility of composite spectra resulting from mixtures of the above conformational states.

It should now be useful to monitor not only fluorescence but also circular dichroism responses of the tetracyclines during membrane Ca and Mg movements accompanying cellular function. The CD spectra of CTC provide a clearer distinction between the effects of different cations, such as Ca and Mg, than is possible by other spectroscopic methods. Recent developments in fluorescence and circular dichroism instrumentation also allow for subsecond time resolution, making possible rapid kinetic measurement of membrane Ca and Mg translocation.

Registry No. Chlortetracycline, 57-62-5; calcium, 7440-70-2; magnesium, 7439-95-4; lanthanum, 7439-91-0.

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Cyclic Nucleotides Modulate the Release of [^{3}H]Adenosine Cyclic 3′,5′-Phosphate Bound to the Regulatory Moiety of Protein Kinase I by the Catalytic Subunit of the Kinase†

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ABSTRACT: The rate of release of bound c[^{3}H]AMP from the two types (A and B) of cAMP binding sites on the regulatory subunit dimer (R_2^I) of rabbit muscle protein kinase I was studied in the presence of the catalytic (C) subunit of protein kinase. Rebinding of released c[^{3}H]AMP was avoided by using highly diluted reactants or adding unlabeled cAMP or its analogues. No significant C-induced dissociation of R_2^I-(c[^{3}H]AMP)$_4$ occurred in the absence of Mg^{2+}-ATP. Of the two options that one or two molecules of C are required to induce the release of c[^{3}H]AMP bound to R_2^I, only the first one was compatible with the first-order dependence on [C] of the rate of release of c[^{3}H]AMP observed over a wide range of C concentrations. In the absence of added unlabeled cyclic nucleotide, the rate of the C-induced release of c[^{3}H]AMP was the same from site A and site B. The apparent second-order rate constant for the association of C to R_2^I(c[^{3}H]AMP)$_4$ was 6×10^6 M^{-1} s^{-1} (37 °C, 0.15 M KCl). Raising the concentration of unlabeled cAMP in the medium up to 1 μM decreased by up to 50% the rate of the C-induced release of bound c[^{3}H]AMP from both sites. This is explained by assuming that the association of one molecule of C to R_2^I(c-[^{3}H]AMP)$_4$ leads to the release of c[^{3}H]AMP first from one R subunit and subsequently, by a process that can be blocked by about 1 μM cAMP, from the other R subunit. A further rise of the cAMP concentration decreased the rate of release from site B only, so that the C-induced release of c[^{3}H]AMP occurred almost exclusively from site A at very high concentrations of cAMP. This suggests that c[^{3}H]AMP is released first from site A and that this vacant site by interacting with cAMP inhibits the release of c[^{3}H]AMP from site B of the same R subunit. The role of site A in controlling the C-induced release was further supported by the finding that several cAMP analogues inhibited the release with potencies correlating with their affinities for site A. The C-induced release of c[^{3}H]AMP from aged R_2^I was about 10 times slower than that from fresh R_2^I. No significant C-induced release of c[^{3}H]AMP was observed from the monomeric fragment obtained by limited trypsin treatment of R_2^I.

The cAMP-dependent protein kinase (cAK)[1] exists in two isozyme forms (cAKI and cAKII), both of which are composed of a regulatory subunit dimer and two catalytic subunits. Activation of the enzyme by cAMP is accompanied by dissociation (Krebs, 1972) according to the overall equation:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C \quad (1)$$

where R_2 is the regulatory subunit dimer and C the catalytic subunit of cAK (Corbin et al., 1978; Weber & Hilz, 1979).

It is known that R_2^I (Døskeland, 1978) and R_2^{II} (Rannels & Corbin, 1980a; Øgreid & Døskeland, 1980) have two types of cAMP binding sites, termed A and B (Døskeland & Øgreid, 1981) according to the rate with which they exchange bound labeled cAMP.

The present study is concerned with the reverse reaction of eq 1. The primary aims were to find the order in which cAMP dissociated from site A and B in the process of the C-induced release of cAMP from its complex with R_2^I and the possible significance of interactions between the binding sites in this process. Another concern was whether one or, as commonly assumed (Smith et al., 1981; Builder et al., 1981; Flockhart & Corbin, 1982), two C subunits must bind to the R_2^I(cAMP)$_4$ complex to effect the release of any bound cAMP.

Experimental Procedures

Materials. c[8-^{3}H]- or c[5′,8-$^3H_2$]AMP (of specific activities varying from 26 to 60 Ci/mmol) and [γ-^{32}P]ATP were from the Radiochemical Centre, Amersham, U.K. Adenosine, AMP, ADP, ATP, cAMP, cIMP, cGMP, and N^6-monobutyryl-cAMP were from Sigma Chemical Co., St. Louis, MO. 8-Amino-cAMP and 2-*n*-butyl-cAMP were kindly supplied by Dr. Jon P. Miller and Dr. R. H. Suva, SRI-International, Menlo Park, CA.

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[1] Abbreviations: cAKI, cAMP-dependent protein kinase, isozyme form I; cAKII, cAMP-dependent protein kinase, isozyme form II; R_2^I, the regulatory subunit dimer of cAKI; R_{t1}^I, partially proteolyzed monomeric fragment of R_2^I; R_2^{II}, regulatory subunit dimer of cAKII; C, catalytic subunit of cAKI or cAKII; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; k_a, apparent second-order rate constant for the association between two molecules; k_d, apparent first-order dissociation rate constant for the dissociation of a complex; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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