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LIPID MONOLAYER EXPANSION BY CALCIUM-CHLOROTETRACYCLINE AT THE AIR/WATER INTERFACE AND, AS INFERRED FROM CELL SHAPE CHANGES, IN THE HUMAN ERYTHROCYTE MEMBRANE

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Chemically induced shape changes of the human erythrocyte may result from cell membrane bending by surface tension changes at the lipid bilayer (Evans, E.A. (1974) Biophys. J. 14, 923–931) implicating differential expansion of the monolayers coupled to form the red cell membrane (Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4457–4461). Interacting with calcium, the antibiotic chlorotetracycline (CTC) transforms crenated cells (echinocytes) into cup-shaped ones (stomatocytes), presumably expanding thereby the red cell membrane inner leaflet relative to the outer one (Behn, C., Lübbemeier, A. and Weskamp, P. (1977) Pflügers Arch. 372, 259–268). Whether the Ca-CTC interaction with lipid monolayers may in fact expand the latter, has now been examined by surface tension measurements at the air/water interface. CTC and lipids appeared to compete for the available sites at the air/water interface, contributing additively to its surface pressure. Ca increased both the adsorption rate of the antibiotic to the interface and the CTC-induced surface pressure increment. The latter was not influenced by the subphase pH and ionic strength, or by the type of phospholipid polar head. Correspondingly, CTC-induced cell shape changes should be determined by the p*Ca* values facing either monolayer of the erythrocyte membrane. Both stomatocytes and echinocytes could indeed be obtained with 0.5 mmol·l^{-1} CTC, the cell shape depending on whether the external medium was adjusted respectively to p*Ca* 9 or to p*Ca* 3. Fluorescence microscopy revealed the Ca-CTC complex to be mostly restricted to the cell in stomatocytes and to the external medium in echinocytes. The possibility of inducing alternative cell shapes by varying the transmembrane Ca-CTC distribution, and the demonstration of a Ca-dependent expansion of even relatively compressed lipid monolayers by CTC, together suggest that the Ca-CTC complex may also differentially expand either leaflet of the red cell membrane.

Introduction

The shape of a cell may express intramembrane mechanics. In 1968 Deuticke [1] conceived the biconcave disk form of the human erythrocyte, as the result of antagonic forces, respectively leading to crenated echinocytes and to cup-shaped stomatocytes. The opposed forces reversibly altering the equilibrium shape were later on thought to act by expanding either monolayer in the red cell membrane [2]. In membrane bilayers, the surface area adjustment tend to be accomodated by curva-

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Abbreviations: CTC, chlorotetracycline; Mes, 4-morpholine-ethanesulfonic acid.

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ture production. Comparatively small alterations of the interfacial chemical free energy density (surface tension) may lead to pronounced cell shape changes [3]. Geometrical calculations show, that above cell shape transformations occur in relation to rather discrete modifications (less than 1% of cell surface) of the difference in area between the outer and inner monolayer [4].

If single monolayer expansion causes specific changes in cell shape, the latter could be indicative of drug interaction with either side of the membrane. Using the antibiotic chlorotetracycline [5] as an amphiphilic calcium probe [6–12], Behn et al. [10] inferred from cell shape changes that the divalent cation is asymmetrically distributed in the red cell membrane. It has now been examined whether the assumption of a Ca-dependent membrane leaflet expansion by CTC may be verified in a model system of single monolayers. CTC indeed increased the surface pressure of an air/water interface, the effect depending on the subphase p*Ca* and on the amount, but not on the nature of the phospholipids added to the interface. The antibiotic remained effective in expanding monolayers up to surface pressure values thought to prevail in the intact erythrocyte. As derived from CTC-induced cell shape changes, the antibiotic also expanded either monolayer in the intact erythrocyte, depending on transmembrane Ca distribution. The latter also determines CTC distribution at the red cell membrane as visualized in fluorescence experiments. Thus it may be suspected that the Ca-dependent cell shape changes induced by the antibiotic in the human erythrocyte may indeed be due to differential surface pressure increases at either monolayer of the red cell membrane. Parts of the present work have been published in abstract form [13].

Materials and Methods

Reagents. CTC was obtained as Aureomycin from Lederle, Munich. Only freshly prepared solutions were used. The p*Ca* values of the subphase solution (100 mmol · l^{-1} NaCl, 50 mmol · l^{-1} Tris-HCl), were calculated using an apparent dissociation constant of CaEGTA of $3.3 \cdot 10^{-7}$ (pH 7.4) and $2.0 \cdot 10^{-5}$ (pH 6.0), following Weber et al. [14]. Phosphatidylcholine and phosphatidylethanolamine were isolated from egg yolk [15] and purified by preparative thin-layer chromatography (TLC). Phosphatidylserine was obtained from Sigma Chemical Co., St. Louis, MO, and purified by TLC. The phospholipid samples were stored under nitrogen at −20°C in chloroform. Total lipid extracts from erythrocyte membranes were obtained from ghosts prepared by osmotic lysis according to Dodge et al. [16]. Extractions were performed with a chloroform/methanol mixture (2:1, v/v) in the presence of butylated hydroxylated toluene, under nitrogen. After filtration, the extract was evaporated under reduced pressure and the lipids redissolved in chloroform/methanol. Non-lipid contaminants were separated with the method of Folch et al. [17]. Polar lipids were separated and determined by two-dimensional TLC according to Rouser et al. [18]. The phospholipid spots were identified using specific reagents [19]. Chloroform or chloroform/methanol (2:1) solutions of lipids and lipid/CTC mixtures constituted the monolayer spreading solutions.

Surface tension. The surface tension of an air/water interface of a fixed area (42.5 cm^2), was measured with the Wilhelmy plate (platinum) modification of the Langmuir film balance. A Cahn RG electrobalance was used, coupled to an *X-Y* Servogor recorder. The monolayer spreading solutions were placed at the air/water interface with a Hamilton microsyringe. Small fluid volumes were added to the continuously stirred subphase, without disturbing the monolayer.

Red cell shape. Human erythrocytes were observed by light microscopy while exposed to various experimental solutions in a perfusion chamber, basically as described before [10]. A 5 μl sample of capillary blood obtained by finger puncture was pipetted (Eppendorf) into 1.5 ml saline solution (100 mmol · l^{-1} NaCl and 50 mmol · l^{-1} Tris-HCl, pH 7.4 and p*Ca* 9, 6 or 3). The blood cells were homogeneously suspended in the saline by a brief and gentle manual shake of the microtest-tube (Eppendorf) containing them. The cell suspension was then placed on a poly(L-lysine) (Sigma P 0879)-treated coverglass [10], where the cells were allowed to sediment for about 20 min, while the preparation was maintained in a humid environment (petri dish). After removing the fluid excess on the coverslip, the latter was attached with a

dental wax/vaseline mixture (1:1) to a metallic frame constituting the central part of a microperfusion chamber. The opposite side of this chamber was sealed by another, albeit untreated, coverslip fastened to the frame with nail polish. The volume contained by the thus completed microperfusion chamber, (0.68 ml) was exchanged about once every 3 min through a closed-circuit perfusion system (two-way perfusion pump, Labotron, Gelting). A laminar saline flow was thus installed with either p*Ca*, the antibiotic being added to a final concentration of 0.5 mmol · l^{-1} after the first 5 min of perfusion had been completed. The microperfusion chamber was held on the stage of an inverted microscope (Diavert, E. Leitz, Wetzlar) equipped with Nomarski interference contrast optics (NP1 40/0.65 P objective) and with an automatic camera (Orthomat, E. Leitz) triggered with a time-driven starter. In serially taken photomicrographs, cell shape changes were evaluated by counting those cells exhibiting one or more spicules (echinocytes), as compared to smooth-shaped cells (discocytes and stomatocytes).

Ca-CTC fluorescence. Red cells prepared as for observation of shape changes (see above) were exposed to 0.5 mmol · l^{-1} CTC in p*Ca* 9 or p*Ca* 3 saline and examined for Ca-CTC fluorescence on the Diavert microscope, the interference contrast optics being replaced by a Heine phase contrast condensor and a Pv Apo Oel 90/1.32 n objective. Cell shape was assessed in this case by transillumination of the preparation with a 6 V 60 W lamp. Membrane-associated Ca-CTC fluorescence [6–12,32], on the other hand, was elicited in the dark field with incident light (HBO 50W/AC lamp, Osram) passing through a Leitz vertical illuminator in position 2 for violet excitation (2 mm BG3 filter) and in position 3 (CBS510 dichroic mirror and LP530 barrier filter) for visual observation and photomicrography of the preparation. For the latter, a high-sensitivity film (Kodak TRI-X Pan, 400 ASA) was used.

Results

Surface tension

Addition of CTC to the subphase of a lipid monolayer reduced the surface tension of the latter, i.e., increased its surface pressure, depending on the presence of Ca (Fig. 1). The lowering of surface tension by CTC, before Ca was added, turned out to be smaller (upper trace) than that seen when the antibiotic acted in the presence of the divalent cation (lower trace). The latter effect was partially reversed by EGTA (Fig. 1). Surface tension lowering by CTC was also enhanced by Mg (unpublished results). The divalent cation effect has, however, been studied further in relation to Ca only.

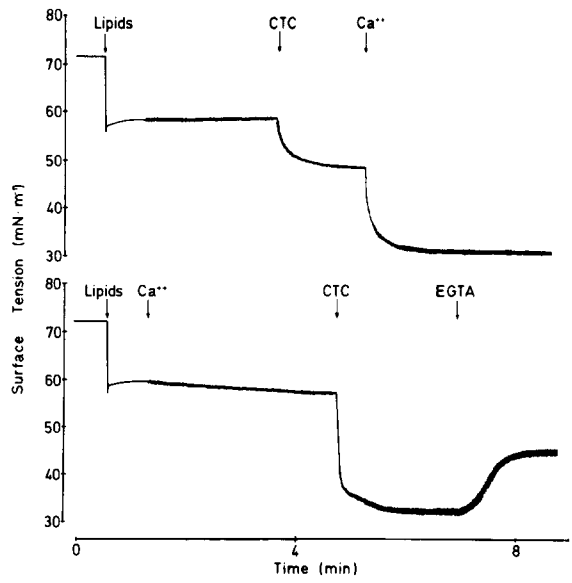


Fig. 1. Surface tension (γ) of an air/water interface as recorded by the Wilhelmy plate method. On an aqueous subphase (100 mmol·l^{-1} NaCl/50 mmol·l^{-1} Tris-HCl) titrated to pH 7.4, a monolayer of total lipids from red cell membranes was placed. Stirring of the subphase began prior to addition of the following reagents (final concentrations): 0.4 mmol·l^{-1} CTC, 0.4 mmol·l^{-1} $CaCl_2 \cdot 2\,H_2O$ and 0.4 mmol·l^{-1} EGTA, as indicated by the arrows. Surface pressure is defined as $\pi = \gamma_W - \gamma_M$, where γ_W = surface tension of the subphase and γ_M = surface tension of subphase + monolayer.

Fig. 2 summarizes the surface pressure change ($\Delta\pi$) induced by CTC at the air/water interface, the latter containing or not containing a lipid monolayer. In the absence of lipids, CTC adsorption to the air/water interface followed a Langmuir-type hyperbolic function [20]. The values of $\Delta\pi_{max}$ and k_p were obtained by linear regression analysis of the data, according to the equation:

$$[CTC]/\Delta\pi = k_p/\Delta\pi_{max} + [CTC]/\Delta\pi_{max}$$

The correlation coefficient was 0.998. In the presence of a lipid monolayer $\Delta\pi_{max}$ was reduced, i.e., less CTC molecules adsorbed to the interface than when no lipid was there (Fig. 2). Conversely, k_p, the constant more related to the kinetics of adsorption, appeared to be unchanged by the presence of a lipid monolayer. In the following experiments, CTC was used at a concentration (0.33 mmol $\cdot$ l^{-1}), which did not saturate the monolayer over a wide range of surface pressures.

The rate of interaction of CTC with the interface ($d\pi/dt$), and the surface pressure change ($\Delta\pi$) induced by the antibiotic, are quantified in Table I. At a given p*Ca*, $d\pi/dt$ scarcely changed when a lipid monolayer was added to the interface. By doubling the lipid surface pressure from 11 mN $\cdot$ m^{-1} to 22 mN $\cdot$ m^{-1}, $d\pi/dt$ decreased slightly. Thus, no major interaction between CTC and lipids appeared to occur. On the other hand, $d\pi/dt$ was strongly Ca-dependent; lowering p*Ca* from 9 to 3, increased $d\pi/dt$ about 30-times. Ca also increased the $\Delta\pi$ induced by the antibiotic. At a given p*Ca*, $\Delta\pi$ was inversely related to the initial surface pressure, but for a given surface pressure value, $\Delta\pi$ was increased by lowering p*Ca* (Table I).

The rise in surface pressure when adding 0.33 mmol $\cdot$ l^{-1} CTC to the subphase, is shown for various conditions in Fig. 3. With increasing amounts of lipids at the interface, $\Delta\pi$ decreased according to a biphasic linear relation. At least in its initial steep segment, the curve seemed to be equally well fitted at p*Ca* 9 by points corresponding to different phospholipids. Thus, the polar head of phospholipids does not appear to play an important role in CTC adsorption to the interface. To test the possibility of ionic interactions between CTC and lipid monolayers, the ionic strength was varied from 0.18 to 0.38 at p*Ca* 6, but no effect on $\Delta\pi$ was observed; it also made no difference whether the subphase was maintained at pH 6 or at pH 7.4. Conversely, by increasing Ca concentration in the subphase, the curves were displaced to the right. The divalent cation thus appeared to increase the lipophilic character of the antibiotic, the effect of the latter on surface pressure remaining apparent well above of 40 mN $\cdot$ m^{-1} at p*Ca* 3 (Fig. 3).

Whether CTC interacts with phospholipid polar head groups was further examined by assessing the

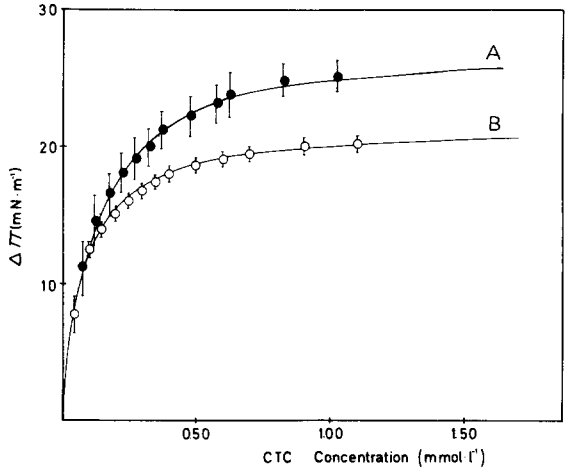


Fig. 2. Surface pressure changes by CTC. The aqueous subphase as in Fig. 1, was calcium-buffered to p*Ca* 6 with EGTA; ○, total lipids of the red cell membrane applied as chloroform/methanol solution to the air/water interface up to a surface pressure of 11.7 mN $\cdot$ m^{-1}; ●, air/water interface without lipids. The points represent means $\pm$ S.D. ($n = 5$). The curves were drawn according to the following equations:

$$\Delta\pi = \frac{(27.7 \pm 0.7)[CTC]}{(0.12 \pm 0.03) + [CTC]} \quad (A)$$

$$\Delta\pi = \frac{(20.7 \pm 0.9)[CTC]}{(0.08 \pm 0.01) + [CTC]} \quad (B)$$

TABLE I

CHLOROTETRACYCLINE ADSORPTION TO THE AIR/WATER INTERFACE HOLDING OR NOT HOLDING A LIPID MONOLAYER

Subphase: 100 mmol $\cdot$ l^{-1} NaCl/50 mmol $\cdot$ l^{-1} Tris-HCl (pH 7.4). Added: 0.33 mmol $\cdot$ l^{-1} CTC. π is lipid surface pressure.

p*Ca*	π (mN $\cdot$ m^{-1})	$\Delta\pi$ (mN $\cdot$ m^{-1})	$d\pi/dt$ (mN $\cdot$ m^{-1}/s)
9	0	14.8	0.24
	11.3	10.5	0.42
	21.8	4.3	0.10
6	0	19.9	2.53
	11.7	13.2	2.25
	22.4	8.9	1.71
3	0	33.2	6.05
	11.7	18.7	6.82
	22.6	15.6	5.65

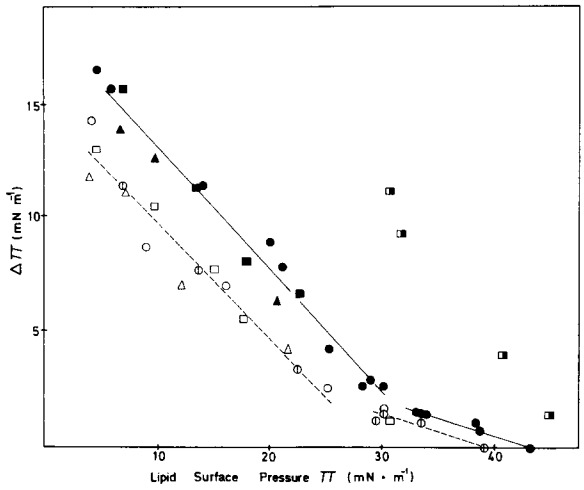


Fig. 3. CTC-induced surface pressure changes as a function of the lipid surface pressure initially present. Each symbol represents a different lipid monolayer and/or subphase. Unless specified, conditions were the same as for ⦶, the monolayer being formed with total lipids of red cell membranes and the subphase containing salts as in Fig. 1, at an ionic strength of 0.18 and p*Ca* 9; ○, phosphatidylethanolamine; □, phosphatidylserine; △, phosphatidylcholine; ■, p*Ca* 6, 300 mmol·l^{-1} NaCl, i.e., ionic strength=0.38; ▲, p*Ca* 6, 50 mmol·l^{-1} Tris/Mes, pH 6; ●, p*Ca* 6. Filled points represent data from total lipids of red cell membranes. The straight continuous line at the left was obtained by calculating the linear regression of the filled point values corresponding to data recorded at lipid surface pressures up to 30 mN·m^{-1}. The equation of this regression line is $y=18.5-0.54x$ ($n=16$, $r=0.99$). The filled point values above 30 mN·m^{-1} could be fitted with another linear regression, according to the formula $y=6.45-0.15x$ ($n=6$, $r=0.97$). The open points (p*Ca* 9) up to 25 mN·m^{-1} of lipid surface pressure are fitted by the broken line at the left, the corresponding equation being $y=14.8-0.50x$ ($n=15$, $r=0.96$). Regression analysis of the open-point values above 25 mN·m^{-1} led to the equation of $y=6.1-0.15x$ ($n=6$, $r=0.87$); ◨, p*Ca* 3, total lipids of red cell membranes.

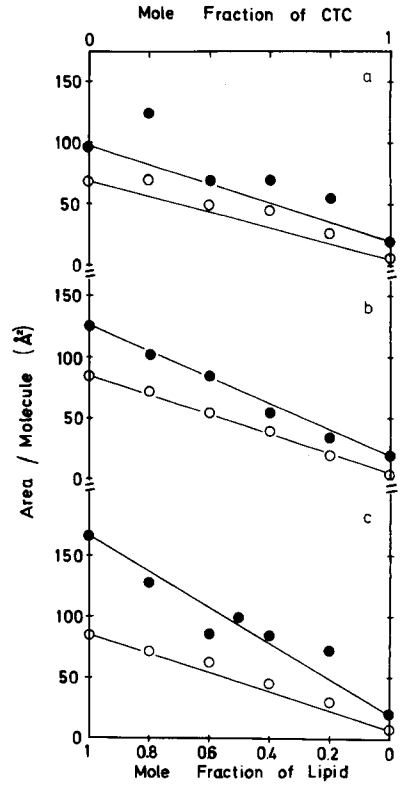
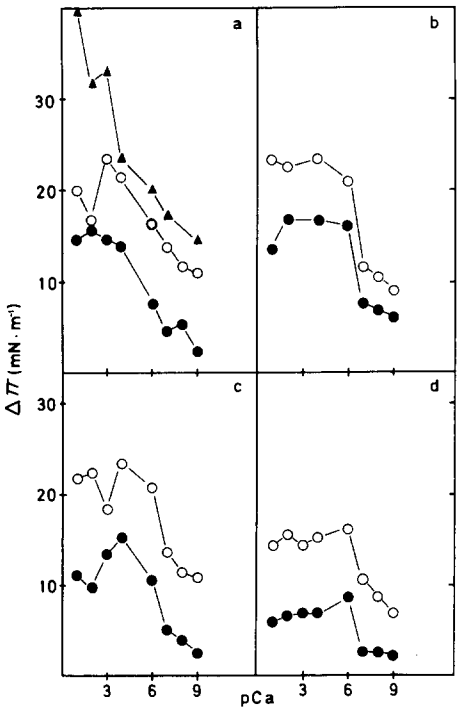


Fig. 4. Average area/molecule of phospholipid-CTC monolayers at two different surface pressures. The continuous line is the additivity rule line. The phospholipid surface pressures (mN·m^{-1}) were: (a) phosphatidylethanolamine, 10.5 (●) and 26 (○); (b) phosphatidylcholine, 13 (●) and 27.5 (○); (c) phosphatidylserine, 8.5 (●) and 23 (○).

area per molecule in monolayers formed with mixed solutions of the antibiotic and phospholipids. At two different surface pressure values and for three phospholipids tested, the area per molecule at the interface closely followed the line representing an independent and additive contribution of the two species to the monolayer (Fig. 4). A slight positive deviation (expansion of the monolayer) was observed for phosphatidylethanolamine, especially at a low surface pressure (10.5 mN·m^{-1}). Calcium did not affect the curve-type shown, although higher area/molecule values were obtained when virtually no Ca was present in the subphase (p*Ca* 9), the corresponding data not being shown here.

The effect of Ca on $\Delta\pi$ is more closely depicted in Fig. 5. Depending on the Ca concentration in the subphase, the CTC-induced surface pressure increase was augmented both in the absence as well in the presence of lipids at the interface, considering either total lipids of the red cell membrane (Fig. 5, upper left) or individual phospholipids (Fig. 5, remainder). The p*Ca* required for reaching saturation pressure in phospholipid monolayers was shifted to higher values as compared to those needed for saturating films of total lipids (Fig. 5). Monolayers of total lipids possibly adsorb less Ca, at a given subphase p*Ca*, than those formed with single phospholipids. For the



points near p*Ca* 3 in Fig. 5 the appearance of precipitates should also be considered. Presumably, it corresponds to Ca-CTC complex formation, the interpretation of the corresponding points, thus becoming further complicated.

Red cell shape

Exposing the glass-adhered erythrocytes to a laminar flow of a nearly Ca^{2+}-free saline turned their smooth, biconcave disk shape into a spicu-

Fig. 5. CTC-induced surface pressure changes at various calcium concentrations. To the subphase, as in Fig. 1, CTC was added up to a final concentration of 0.33 mmol·l^{-1}, after the lipid monolayer had been formed at the interface. The CTC-induced increase in surface pressure was recorded under the following conditions: (a) no lipids added (▲); red cell membrane lipids added up to a surface pressure π of 10.5 mN·m^{-1} (○); idem, but surface pressure reaching 22.5 mN·m^{-1} (●); (b) phosphatidylcholine, $\pi = 10$ mN·m^{-1} (○) and $\pi = 21.5$ mN·m^{-1} (●); (c) phosphatidylethanolamine, $\pi = 10$ mN·m^{-1} (○) and $\pi = 21.5$ mN·m^{-1} (●); and (d) phosphatidylserine, $\pi = 11$ mN·m^{-1} (○) and $\pi = 21$ mN·m^{-1} (●).

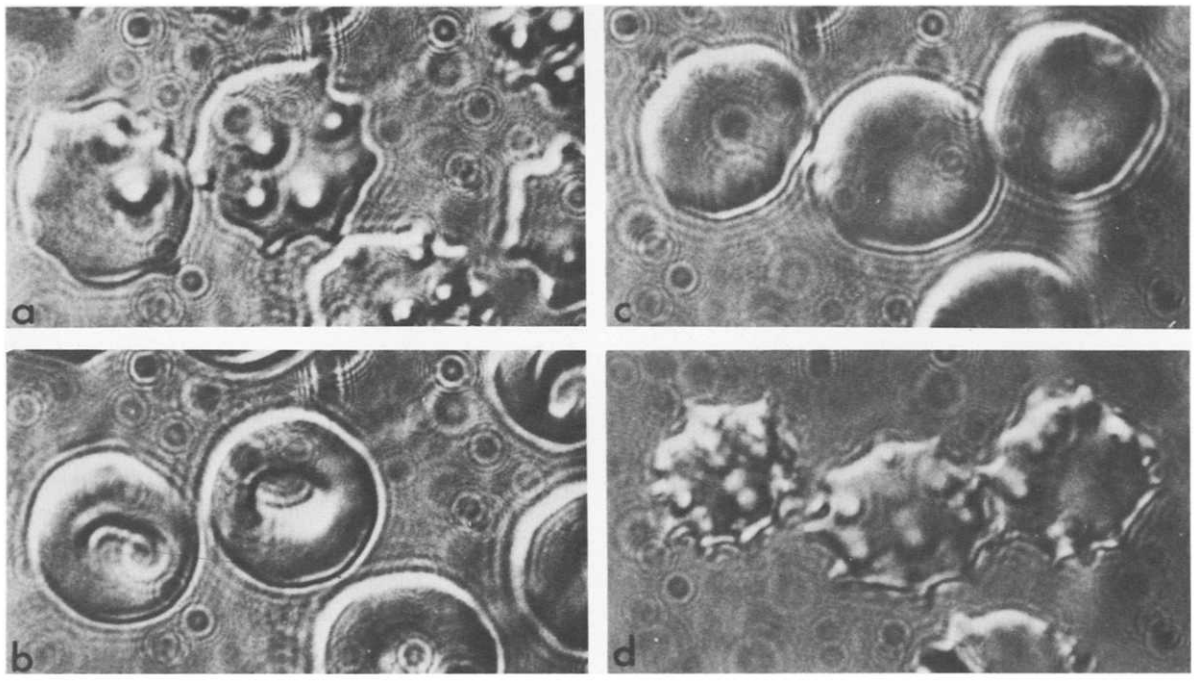


Fig. 6. CTC-induced shape changes in superfused red cells at different p*Ca* values. (a) p*Ca* 9; (b) p*Ca* 9 plus 0.5 mmol·l^{-1} CTC; (c) p*Ca* 2; (d) p*Ca* 2 plus CTC as in (b). At a given p*Ca* the photomicrographs represent the same cells immediately before CTC treatment (above) and 10 min after the antibiotic has been added to the perfusion fluid (below).

TABLE II

CALCIUM-DEPENDENT EFFECTS OF CTC ON HUMAN ERYTHROCYTE SHAPE

Percentage of crenated cells (mean ± S.E.) among erythrocytes superfused for 10 min with saline ($n = 3$). About 200 cells were classified in each experiment.

p*Ca*	Controls	0.5 mmol·l^{-1} CTC
9	72.8 ± 3.9	3.3 ± 1.9
6	16.5 ± 12.7	0.5 ± 0.5
3	2.5 ± 0.8	73.3 ± 5.8 [a]

[a] $n = 2$.

lated (crenated) one (Fig. 6, upper left). Crenated erythrocytes (echinocytes) appeared independently, whether the adjacent glass surface was polylysine-treated or not, as long as the cells were rather strongly exposed to the Ca^{2+}-free saline i.e., when they were either superfused, washed or even shaken (at very low hematocrit, see Materials and Methods) in this medium (see also Ref. 10). This echinocytogenic effect was progressively lost as the Ca concentration in the perfusate was increased (Fig. 6, upper right; see also Table II). Adding CTC to the low Ca^{2+} medium transformed the crenated echinocytes into cup-shaped stomatocytes (Fig. 6, lower left; Table II). Conversely, in a high Ca^{2+} medium, the antibiotic transformed the smooth cell shape into a crenated one (Fig. 6, lower right; Table II). Thus, CTC acted as a stomatocytogenic agent when Ca was nearly absent in the external medium (p*Ca* 9) and as an echinocytogenic one, if the external Ca concentration approximated physiological levels (p*Ca* 3). If the bilayer couple concept [2] applies, CTC expands either monolayer of the red cell membrane, depending on the respective calcium content.

Ca-CTC fluorescence

Excitation-emission conditions appropriate to reveal membrane-associated Ca-CTC [6–12,32] indicated the relative content of the complex to be larger in stomatocytes than in echinocytes (Fig. 7). Whereas echinocytes appeared to be excluded from the lightly fluorescent medium, stomatocytes exhibited a distinct fluorescence at the cells surface. Differences in cellular CTC content may implicate

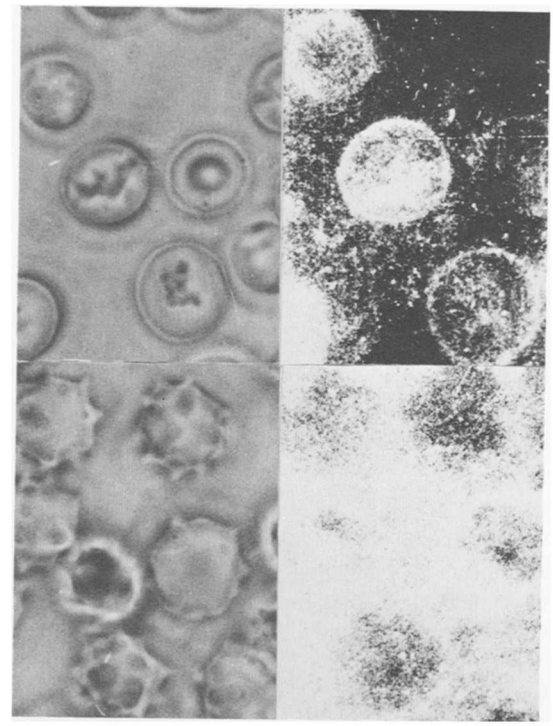


Fig. 7. CTC-fluorescence as related to cell shape. Photomicrographs of human erythrocytes examined by phase contrast for cell shape (bright field) at the left and for CTC-fluorescence (dark field) at the right, while exposed to 0.5 mmol·l^{-1} CTC either at p*Ca* 9 (upper row) or at p*Ca* 3 (lower row). The fluorescence photomicrographs, originally taken on high sensitivity film, were rephotographed for contrast enhancement on documental film (Kodalith).

intramembrane distribution patterns which allow the antibiotic to interact mainly with the inner monolayer in stomatocytes and with the outer one in echinocytes.

Discussion

Calcium increased the affinity of CTC for the air/water interface containing or not containing a lipid monolayer. Correspondingly, in biological membranes, a selective adsorption of the antibiotic to either membrane leaflet, depending on the respective Ca content, was expected. Cell shape changes and the distribution of Ca-CTC fluorescence in intact erythrocytes appear to support this prediction.

Surface tension

The lipid monolayer per se reduced the adsorption of CTC to the interface (Fig. 2 and Table I). This is the opposite of what occurs with the adsorption of a positively charged protein (cytochrome *c*), which is facilitated by the presence of lipids [21]. The surface pressure increase induced by CTC in a monolayer at a given Ca concentration depended on the amount of lipids present there, i.e., on the initial surface pressure (Fig. 3), but not on the polar head of a particular phospholipid. The merely additive contribution of lipids and CTC to surface pressure was independently demonstrated when mixing CTC and phospholipids in different proportions (Fig. 4).

Adsorption of CTC to the interface did not depend on electrostatic interactions, as indicated by the failure to influence it by doubling the ionic strength or by lowering the subphase pH from 7.4 to 6 (Fig. 3). Membrane binding of anionic and cationic amphiphiles is, by contrast, respectively increased and decreased by an augmentation of ionic strength [22]. Thus, CTC may not be very polar under the conditions described. CTC has been reported to have a partial negative charge at pH 7.4 (cf. Ref. 6), but at least in the range between pH 7 and 10 and in the presence of Ca, neutral and differently charged forms of both the complexed and the uncomplexed compound may coexist [12]. In the present experiments, where CTC adsorbed to lipid monolayers less in relation to charges than to the presence of Ca, the antibiotic may preferentially act as a neutral complex when approaching the interface.

The presence of Ca in the subphase markedly influenced CTC adsorption to the air/water interface, as shown both by equilibrium values of surface pressure increase (Table I and Figs. 3 and 5) and by adsorption kinetics (Table I). The divalent cation facilitated the adsorption of CTC to the interface, irrespective of the type of phospholipid present there (Fig. 5). Correspondingly, lipid asymmetry in biological membranes (for a recent review see Ref. 23), would not influence CTC distribution across their structure, as much as would Ca (see discussion on cell shape). The mechanism by which Ca increased CTC adsorption to a lipid monolayer ($d\pi/dt$ augmented by about 30-times when p*Ca* was changed from 9 to 3, as shown in Table I), may involve specific conformation changes of the antibiotic upon Ca binding [24]. A more general effect of divalent cations cannot be excluded, however, since preliminary experiments indicate that CTC adsorption to the air/water interface is increased also by Mg (unpublished data).

CTC adsorbed to the interface up to a surface pressure of about 40 $mN \cdot m^{-1}$ at p*Ca* 6 and to even higher values at p*Ca* 3 (Fig. 3). These values clearly exceed the monolayer surface pressure of 31–35 $mN \cdot m^{-1}$ as found by Van Deenen et al. [25] to be representative for the outer leaflet of the red cell membrane, considering the susceptibility of this structure to phospholipases which to be effective require a rather tightly compressed lipid layer [25]. Interestingly, the maximum of calcium adsorption to lipid monolayers (phosphatidylserine and phosphatidylinositol) also occurs at a film pressure of about 30 $mN \cdot m^{-1}$, corresponding to optimal spacing between phospholipid molecules for two-point electrostatic attachment of the divalent cation [26]. Thus, in spite of the large 'internal pressure' assumed to prevail in biological membranes [27,31], the calcium-complexing, amphiphilic agent CTC may be able to expand lipid monolayers also in the intact cell.

Red cell shape

The findings that (a) lipid monolayers could be expanded by CTC at surface pressure values similar and above to those detected in red cell membranes and that (b) adsorption of the antibiotic to lipid monolayers was increased by the presence of Ca in the subphase, may be used to interpret the CTC effects on red cell shape.

According to the so-called bilayer couple hypothesis, differential, although not necessarily equivalent [28], surface area expansions of either monolayer in the red cell membrane may account for shape changes of the human erythrocyte [2,28,29] and possibly also for control of some general membrane functions like ion transport [30]. Though presently questioned at the laboratory [27,31] where first formulated [2], the bilayer couple approach can be used here to discuss the observed cell shape changes in the light of the surface tension changes induced by CTC in artificial monolayers.

CTC reversed the crenated cell shape to the cupped form (stomatocytes), provided that Ca was nearly absent in the external medium (Table II, Fig. 6 and Ref. 10). The Ca content of the inner monolayer presumably being higher than that of the outer monolayer (in a cell exposed to p*Ca* 9), would be a plausible reason for the amphiphilic Ca-complexing agent CTC to distribute accordingly. A corresponding expansion of the inner monolayer, compensating for the already expanded outer leaflet, or exceeding it, would succesively lead to discocytes and stomatocytes. Conversely, at high external Ca concentration (p*Ca* 3), where the outer monolayer may contain (adsorb) more Ca than the inner one, CTC just induced crenation, this shape transformation being otherwise (p*Ca* 9) reversed by the antibiotic. CTC thus preferentially would expand that side of the membrane facing the relatively higher Ca concentration. Exactly this would have been expected on the basis of the behavior of the antibiotic at the air/water interface (see above).

Expansions of either monolayer in the red cell membrane have been hitherto induced (if at all) by various probes, mostly ionic ones, distributing themselves according to fixed asymmetries across the membrane. In the present work, however, both monolayers appeared capable of being expanded by the same probe (CTC), but differently, depending on transmembrane Ca distribution. This would also confirm the use of CTC for determining the distribution of Ca in the red cell membrane [10].

Although effective in expanding lipid monolayers at the air/water interface, CTC may not necessarily do the same in a non-planar, protein-entangled bilayer like the red cell membrane. Even if this were so, additional Ca-dependent interactions between the antibiotic and the membrane might occur, being eventually also responsible for drastic changes in cell shape. Thus, a potential ionophoretic character of CTC [32] should also be considered as a possible shape-modifying factor. The Ca-ionophore A23187 leads to crenation in the presence but not in the absence of calcium [33], similar to the case of CTC (Fig. 6 and Table II). Conversely, however, to the irreversible nature of the A23187-induced crenation [34], both the echinocytogenic and the stomatocytogenic effects of CTC can be reversed by appropriately washing the cells (Ref. 10 and unpublished results). Whether CTC, on the other hand, may influence cell shape by variable interaction with Ca-induced and transamidase-catalyzed cross-linking of membrane proteins [33] remains to be determined.

Beside a conceivable role of cytoskeletal proteins (spectrin) in cell shape control (Refs 34, 35, 36; for a recent review see Ref. 37), there may also be some relief of monolayer lipid packing through the complexation by CTC of membrane-associated Ca. The cell-shape sequence echinocyte-discocyte-stomatocyte has been regarded as the expression of successive changes in the membrane's elastic state, where the crenated shape would indicate a strong negative rather than positive intrinsic curvature of the membrane, while at the other extreme the cup-shape of the stomatocyte would supposedly result from an increase in fluidity of the membrane's hydrophobic core [38,39]. Whether CTC influences the physical state of the red cell membrane is unknown.

Anyhow, CTC influences red cell shape. If differential surface tension changes at either bilayer half are implied, the antibiotic should distribute accordingly across the erythrocyte membrane. So-called membrane-associated Ca-CTC can be detected by fluorescence methods [6–12,32], it not being of immediate interest in this respect whether the corresponding signal actually probes divalent cations bound to specific sites in the membrane or whether the latter is probed by the Ca-CTC-complex [12]. Photomicrographs of erythrocytes thus examined for Ca-CTC show the corresponding fluorescence mostly restricted to the cell in stomatocytes and to the external medium in echinocytes (Fig. 7). A higher CTC content throughout the membrane region would more probably imply an expansion of the inner monolayer relative to the outer one than when CTC is preferently confined to the outer side. Cell shape changes induced by a higher content of a foreign molecule (lysophosphatidylcholine) in the outer monolayer, can be balanced by a much lower concentration of this substance in the inner monolayer [28]. The observed variations of cellular CTC content thus may indicate different adsorption of the antibiotic to either monolayer, depending on the respective subphase p*Ca*.

The final mechanism by which CTC reduces

surface tension in artificial and perhaps also in natural membranes remains unknown. Amphipaths do not seem to be properly incorporated into the lipid core of the erythrocyte membrane [27,31,40], presumably because of a high 'internal pressure' in the latter [27,31], the actual site of the interaction of the drug with the cell possibly being the membrane/water interface [40]. CTC can expand even rather tightly compressed monolayers, thus potentially being effective also in the red cell membrane, as has previously been discussed in relation to the results depicted in Fig. 3. The mechanism by which CTC reduces surface tension at an interface, however, may not imply a mere incorporation of the drug into the respective monolayer. Electrostatic interaction of a drug with ionizable groups of lipid polar heads will change the strength of repulsion and correspondingly the order between membrane components [41]. Ionic strength, on the other hand, being without major influence on the CTC effect (Fig. 3), disregards to some extent a role of electrostatic interactions in the present case. Concerning any possible implication of divalent cations in the (various?) mechanisms of action of CTC on membranes, it is remarkable that Ca is displaced from lipid monolayers by other amphiphiles also known to modify the red cell shape [42]. Sequestration and/or complexation of divalent cations may affect lipid as well as protein components of cell membranes [43], presumably also altering the surface tension at the corresponding interfaces.

It may be concluded that Ca-CTC reduces the surface tension of lipid monolayers at the air/water interface under conditions applicable to the red cell membrane. Cell shape changes depending on transmembrane Ca-CTC distribution, moreover, suggest that differential monolayer expansions may occur by the antibiotic also in the intact erythrocyte.

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