

# Control of Erythrocyte Shape by Calmodulin

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**ABSTRACT** Erythrocytes are deformable cells whose shapes can be altered by treatments with a variety of drugs. The forms the erythrocyte may assume vary continuously from the spiny "echinocytes" or crenated cells at one extreme to highly folded and dented "cupped" cells at the other extreme. Examination of 39 compounds for cup-forming activity revealed a remarkable correlation between their ability to form cupped cells and their inhibitory activity against the calcium regulatory protein, calmodulin. Calmodulin is known to interact with several erythrocyte proteins including spectrin, spectrin kinase, and the  $\text{Ca}^{++}$  ATPase calcium pump of the membrane. These proteins regulate the form of the cytoskeleton as well as intracellular calcium and ATP levels. It is proposed that calmodulin is required to maintain normal erythrocyte morphology and that in the presence of calmodulin inhibitors, the cell assumes a cupped shape.

Normal erythrocytes are deformable and exhibit a wide variety of morphologies which are functions of their environment. Bessis and others have described a continuous interchangeable series of erythrocyte forms from spiny "echinocytes" through the familiar biconcave "discocytes" to dented or folded "cupped" cells or "stomatocytes" (3, 10, 40). Any given morphology represents a steady state outcome of a tendency towards echinocytic morphology (crenation) and an opposing tendency towards invaginated forms (cupping). A variety of compounds having cupping and crenating properties have been documented (3, 10, 40) and treatment with combinations of compounds with antagonistic effects generates intermediate forms consistent with a steady state interpretation (10, 39). As a general rule, crenators are neutral or anionic compounds but they have a wide range of chemical structures (10). Many are detergents or have membrane depolarizing properties and may operate via depletion of cellular stores of ATP, which is known to accompany crenation (4, 10). On the other hand, cup formers, with one exception, are all amphiphilic cationic compounds at physiological pH and are structurally related (10, 40). They have homo or heterocyclic ring systems which are frequently halogen or methoxy substituted and attached to the ring(s) is an amine (usually tertiary) containing side chain. The exception, phlorrhizine, is a neutral ring compound with a  $\beta$ -D-glucose side chain.

Explanations of the action of crenators and cup-formers have sought to explain their activities in terms of preferential partitioning of the compounds into the inner or outer lipid leaflets of the plasma membrane. This would expand one

leaflet's area relative to the other and result in shape changes; this model is the "bilayer coupling hypothesis" (39, 40). The preferential distribution of anionic lipids like phosphatidyl serine in the inner membrane leaflet would favor the intercalation of cationic cup formers and conversely the phosphatidyl choline and ethanolamine in the outer membrane leaflet would recruit anionic crenators (44). By this model, nonpenetrating compounds which partition into the membrane would be crenators regardless of charge. Methochlorpromazine, a quaternary amine crenator of intact cells, becomes a cup-former when applied to unsealed erythrocyte ghosts, whereas its tertiary amine analog, chlorpromazine, is a cup former of either intact cells or ghosts. Similar results are obtained with the pairs: lidocaine and QX-222 and HK-27 and HK-25 (39, 40). The differences observed in the action of quaternary vs. tertiary amine analogs on intact cells could be explained by their different abilities to cross the plasma membrane. Similarly, in experiments with pairs of quaternary and tertiary local anesthetics in some nerve preparations, the quaternary analog is only significantly active when applied to the cytoplasmic face of the membrane (13, 23).

Two observations suggest that the intercalation of drugs into the membrane, though important, is not sufficient to explain the morphological changes. First, erythrocytes incubated with wheat germ agglutinin (WGA), which binds to the transmembrane protein, glycophorin, are reversibly immobilized in the echinocytic or discoid morphology which existed before WGA binding. They remain in this form despite changes in environment which would normally cause a discocyte to echinocyte

transformation (or vice versa). However, when the sugar *N*-acetylglucosamine is added to displace the WGA, the cells rapidly undergo the appropriate transitions. This demonstrates that mobility of at least this one protein is required for shape changes to occur (2). Second, Seeman (37) found that erythrocyte membrane expansion induced by local anesthetics and chlorpromazine was ten times greater than can be accounted for on the basis of simple intercalation of the compounds. Recent measurements of amphipath concentrations in membranes by Conrad and Singer (9), using the new technique of hygroscopic desorption, suggest that the older measurements may have overestimated membrane concentrations of these drugs. Franks and Lieb (12) have made recent measurements of drug concentrations in membranes using very sensitive techniques. Their findings addressed the issue of volumes occupied by drug molecules in membranes vs. the aqueous medium and found that there is little difference. Occupied volume is little more than the volume of the drug molecules themselves. This further draws attention to the idea that a specific drug interaction site is responsible for membrane "expansion" rather than a general perturbation. This suggests that a conformational change in membrane proteins or a change in their organization is responsible for the expansion. Cupping compounds might also affect the state of the membrane by perturbing the normal structure of its lipids and displacing membrane-bound calcium (37).

The results reported here, and re-examination of published studies on erythrocyte shape changes in the light of recent work on the pharmacology of the calcium-binding protein, calmodulin, suggest a different molecular mechanism for cupping. Specifically, calmodulin inhibitors are found to be cup-formers and the known cup-formers are either demonstrated calmodulin inhibitors or are structurally very similar and may be characterized as suspected antagonists. This implicates calmodulin as a potential regulator of erythrocyte shape and this idea is developed below.

## MATERIALS AND METHODS

Human erythrocytes were washed with phosphate-buffered saline, pH 7.4 (PBS), and partially depleted of their ATP by overnight incubation at 37°C or 23°C to produce populations of cells enhanced in proportion of echinocytes to facilitate scoring. The cells were cupped by incubating them at room temperature for 15 min at a hematocrit of ~5% in PBS containing the test compound and fixed for at least 15 min with 2% glutaraldehyde in PBS. Cupping was scored using Nomarski differential-interference contrast optics on a Leitz Ortholux II microscope by counting the number of "cupped" cells with characteristic deep concavities, irregular dimples, and deep folds, described by Bessis (3) as stomatocytes, spherostomatocytes, and knizocytes, as well as multiply concave erythrocytes (cf. also Fig. 2). This type of measurement has a subjective component but comparison of 50% effective doses ( $EC_{50}$ 's) between separate experiments was quite reproducible.

Trifluoperazine·2HCl, trifluoperazine sulfoxide·2HCl, chlorpromazine·HCl, chlorpromazine sulfoxide·HCl and prochlorperazine·2HCl were obtained from Smith, Kline and French Laboratories (Philadelphia, PA). Fluphenazine·2HCl was a gift of E. R. Squibb & Sons, Inc. (Princeton, NJ). Thiothixene·HCl was purchased from Pfizer, Inc. (New York, NY), and Chlorprothixene·HCl from Hoffmann-LaRoche (Nutley, NJ) R24571 = 1-[bis-(*p*-chlorophenyl)methyl]-3[2,4-dichloro beta-(2,4)dichlorobenzyloxy)-phenethyl]-imidazolium chloride was purchased from Janssen Pharmaceutica (Beerse, Belgium). W-7 = N-(6-aminohexyl)5-chloro-1-naphthalene sulfonamide and W-5 = N-(6-aminohexyl)-1-naphthalene sulfonamide were obtained from Rikaken Co., Ltd. (Nagoya, Japan). Haloperidol lactate was from McNeil Pharmaceuticals, Inc. (Spring House, PA). All other reagents were from Sigma Chemicals (St. Louis, MO).

## RESULTS

Exposure of erythrocytes to the calmodulin inhibitors: R24571, W7, W5, trifluoperazine, trifluoperazine sulfoxide, chlorpro-

mazine, chlorpromazine sulfoxide, d,l-propranolol and others demonstrated sigmoid dose-dependent induction of cup morphology with 50% effective concentrations ( $EC_{50}$ ) ranging from 2  $\mu$ M to >1 mM (Fig. 1). The order of potency and  $EC_{50}$ 's for cupping closely match the 50% inhibitory doses of these compounds for calmodulin-dependent enzyme activation (14, 15, 20, 21, 22, 32, 46, 48, 49). The phenothiazines and thioxanthenes, fluphenazine, prochlorperazine, chlorprothixene, and thiothixene were also potent cup formers at concentrations inhibitory of calmodulin (78% to >95% cups at 30  $\mu$ M). Especially noteworthy are the relationships of the phenothiazines to their much less calmodulin antagonistic sulfoxide derivatives and the relationship of W7 to its less calmodulin active congener, W5. In every case the inactive (*vis-à-vis* calmodulin) analog was a poorer cup-former than its active analog. Fig. 2 shows representative fields of unfixed erythrocytes before and after incubation with W7 and high magnification views of several cells with typical cupped morphologies.

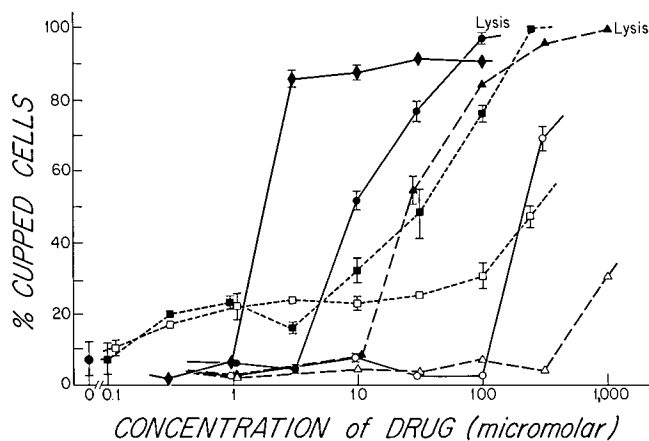


FIGURE 1 Cup-forming activity of calmodulin inhibitors. Seven representative calmodulin inhibitors demonstrate the sigmoid, dose-dependent induction of cupped morphology observed with all 14 drugs examined in this study. Cells were incubated for 15 min in PBS containing test compounds and scored as cupped if their morphology corresponded to those forms defined by Bessis (3) as stomatocytes, spherostomatocytes, knizocytes or multiply concave erythrocytes. 50% effective doses ranged from 2  $\mu$ M to >1 mM and the order of cupping potency was the same as the order of effectiveness in inhibiting calmodulin. Trifluoperazine and chlorpromazine at high concentration caused significant lysis. TFP (●) and TFSO (○) are abbreviations for trifluoperazine and its sulfoxide derivative. CPZ (▲) and CPZSO (△) refer to chlorpromazine and chlorpromazine sulfoxide. R24571 (◆), W7 (■), and W5 (□) are defined in Materials and Methods. Error bars are standard deviations for two to four determinations on at least 200 cells.

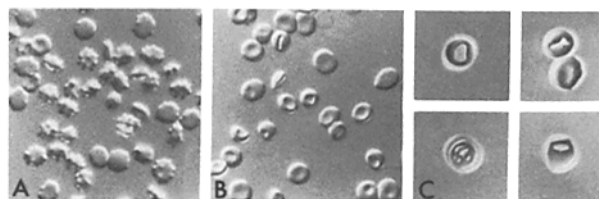


FIGURE 2 Morphology of cupped cells. Nomarski differential-interference contrast photos of unfixed erythrocytes treated with  $1 \times 10^{-4}$  M W7 in PBS. Fields contain typical populations of erythrocytes before (A) and 5 min after drug treatment (B). C illustrates typical cupped forms seen in phase contrast. In A cells are echinocytic because they have been depleted of their ATP to facilitate scoring of morphological differences.

TABLE I  
Anti-CaM, Cup-forming, and Antihemolytic Activities

Name of compound and family	Anticalmodulin activity ID <sub>50</sub> μM and assay	Cupping EC <sub>50</sub> μM	Antihemolysis EC <sub>50</sub> μM
<b>Phenothiazines</b>			
Trifluoperazine	2-10 P (20, 24, 48) C (22, 46, 49) 6-50 D (22, 48, 49) 1-1.5 49)	<u>10</u>	0.97-6 (20, 37, 38)
Trifluoperazine sulfoxide	30-45 D (48, 49)	<u>170</u>	?
Chlorpromazine	30-42 P (20, 48) 135 C (32) 5.3-8 D (48, 49) Yes K (15)	<u>30</u> 10-50 (3, 10, 40)	3-19 (20, 37, 38)
Methochlorpromazine (4° amine analog of chlorpromazine)	?	100 (ghosts) crenates intact erythrocytes (40)	?
Chlorpromazine sulfoxide	2,500 P (20, 48) 170-250 D (48, 49)	<u>&gt;1,000</u>	200 (37)
Fluphenazine	13 P (20)	<u>98% at 30</u> <u>EC<sub>50</sub> ≈ 10</u>	1.5-10 (20, 37, 38)
Prochlorperazine	16 P (20)	<u>96% at 30</u> <u>EC<sub>50</sub> ≈ 10</u>	1.6-6 (20, 37, 38) (eliminated by photolysis)
Promethazine	200-340 P (20, 21, 48) D (48, 49) 40-60	?	20 (20)
Thioridazine	18 P (21)	?	?
<b>Thioxanthenes</b>			
Chlorprothixene ( <i>cis</i> or <i>trans</i> )	16 P (48) Yes C (32)	<u>87% at 30</u> <u>EC<sub>50</sub> ≈ 10</u>	?
Thiothixene ( <i>cis</i> or <i>trans</i> )	30-40 P (48) 7-17 D (48, 49)	<u>78% at 30</u> <u>EC<sub>50</sub> ≈ 10</u>	?
<b>Naphthalene sulfonamides</b>			
W7	28 P (15, 49) 51-80 K (15, 26) 31 D* (15)	<u>35</u>	?
W5	240 P (15, 49) 230 K (15) 210 D* (15)	<u>300</u>	?
<b>Butyrophenones</b>			
Haloperidol	40-60 P (20, 48)	<u>80</u>	0.1-10 (38) 22-65 (20, 37)
Penfluridol	10 P (48)	?	25 (38)
<b>Antiadrenergics</b>			
Propranolol ( <i>d</i> , 1)	180 C (46)	<u>175</u> Yes (10)	250 (37)
Reserpine	130-200 P (48, 49)	Yes (10)	16-20 (37, 38)
<b>Antimalarials</b>			
Mepacrine = quinacrine	56 C (46)	<u>60</u>	?
Primaquine	?	Yes (10, 37)	?
Chloroquine	?	Yes (10)	?
<b>Local anesthetics</b>			
Dibucaine = cinchocaine	180 C (46) 190 P (43) 190 M (43) 220 D* (43)	[300] (10)	100 (36)
Tetracaine	350 C (46) 440 P (43) 1,010 M (43) 920 D* (43)	[300] (10)	50 (36)
Procaine = novocaine	?	Yes (10)	3,500-5,000 (36, 38)
Phenacaine	930 C (46)	?	?
QX-572	310 C (46)	?	?
Lidocaine = xylocaine	4,800 P (43) 6,800 M (43) 5,800 D* (43)	[100] (40)	2,000 (36)

TABLE I—Continued

Name of compound and family	Anticalmodulin activity ID <sub>50</sub> uM and assay	Cupping EC <sub>50</sub> uM	Antihemolysis EC <sub>50</sub> uM
QX-222 (4° analog of Lidocaine)	?	[100] (40) crenator	?
HK-27	?	[100] (40)	?
HK-25 (4° analog of HK-27)	?	[100] (40) crenator	?
Antianginics and vasodilators			
Prenylamine	Yes NA (15, 49)	Yes (10)	15 (37)
Verapamil	No C (18)	100–400 (10)	?
Papaverine	250, 650 D (48, 49)	Yes (10)	?
Others			
R 24571	0.5 C (14)	<u>2</u>	?
Vinblastine	Yes P and C (45) P (47)	20 (17)	?
	16		
Phlorrhizine	?	[250] (10)	?
Benzydamine	?	Yes (10)	?

The antihistamines pheniramine, brompheniramine, and bamipine are also cup-formers, but no calmodulin related data are available (10). All concentrations of drugs are given in micromolar and represent 50% inhibitory or effective doses when referring to enzyme or bioassays and drug displacement. Bracketed values are effective doses which are not necessarily 50% values. For binding assays the value is that for  $K_d$ . Yes and No indicate positive or negative results, but no concentrations are given. Calmodulin assays are for: P, cyclic AMP phosphodiesterase from heart or smooth muscle; C,  $Ca^{++}$  ATPase from erythrocyte membrane; K, myosin light chain kinase from smooth muscle and platelet or calmodulin dependent protein kinase from synaptosomes; D, direct binding of radioactive compound to purified calmodulin or displacement of tritiated trifluoperazine by an unlabeled compound ( $D^*$  is displacement of tritiated W7). NA means no assay was explicitly referred to in the reference and ? means no data are presently available. Underlined values under cupping refer to new data presented here and the assay is described in the text. Antihemolysis refers to protection of erythrocytes against hypotonic lysis and is measured colorimetrically for hemoglobin release. Numbers in parentheses are references.

We have compared 39 compounds for their anticalmodulin, cup-forming and antihemolytic activities where known. Table I summarizes the observations available in the literature and includes new data on the cup forming activity of 14 calmodulin inhibitors. Protection against hypotonic lysis is correlated with the ability of compounds to form cups and with membrane expansion; it is a useful indicator of which compounds, not yet tested for cupping ability, are likely candidates for having this activity (37, 38). Landry et al. (20) have also shown a correlation between membrane stabilization (antihemolytic activity) and anticalmodulin activity for several phenothiazines and a butyrophenone. Antihemolysis is measured by the degree to which erythrocytes are protected by drugs against lysis in hypotonic saline with subsequent release of hemoglobin (measured colorimetrically). Examination of the table reveals a remarkable correlation between the three properties of cupping, antihemolysis, and calmodulin inhibition. The order of potency of the various compounds is similar for the three properties and the effective doses are of the same order of magnitude. Where the data are available, every compound known to be a calmodulin inhibitor was found to be a cup-former. Similarly, where examined, all known cup formers were calmodulin inhibitors with the one exception of verapamil. It is a calcium channel blocker and might prevent calmodulin activity by blocking its access to calcium. Observations on three pairs of quaternary/tertiary amine analogs illustrate the finding that the site of cupping activity is on the inside of the membrane (40). Unsealed ghosts are cupped but intact cells are crenated by the quaternary analog, whereas the tertiary analog cups both ghosts and cells. Fig. 3 presents the structures of the drugs tested and shows the structural similarities between compounds in each family and between groups.

## DISCUSSION

Based on the interactions of calmodulin with erythrocyte cytoskeletal proteins and the action of calmodulin antagonists as

cup formers, we postulate that the control of erythrocyte shape is under regulation by calmodulin. This would result from its direct and indirect effects on the spectrin-actin cytoskeleton and by control of intracellular ATP and  $Ca^{++}$  levels.

A cytoskeleton consisting of the proteins: spectrin, ankyrin (and other syndeins), band 4.1, and actin is connected to the erythrocyte membrane on its cytoplasmic face via the transmembrane protein band 3 (5). Factors which control the assembly, state of polymerization, and organization of these cytoskeletal elements control the cell's shape (5).

Recently it has been demonstrated that calmodulin binds specifically and tightly to spectrin and although the function of this binding has not yet been ascertained, it is likely that it would modulate the state of polymerization or interaction of spectrin with actin and other proteins (42).

Phosphorylation of spectrin band 2 has been correlated with spectrin-actin interaction and morphological transformation from discocyte to echinocyte in erythrocyte ghosts, but this point is somewhat controversial (1, 4, 29, 30). Spectrin phosphorylation is regulated by calcium via a calmodulin-dependent spectrin kinase which is inhibitable in vitro by the commonly used calmodulin inhibitor, trifluoperazine (a potent cup-former) (16). Since calmodulin inhibitors affect spectrin phosphorylation, it is thus likely that they should antagonize echinocyte formation and favor stomatocytes as is actually observed. Calmodulin dependent phosphorylation of several membrane proteins has been demonstrated in a variety of cell types and is not unique to spectrin (35). If the phosphoproteins ankyrin and band 4.1 use such calmodulin regulated kinases they may also be able to regulate red cell cytoskeletal organization in an analogous fashion to spectrin.

The level of intracellular ATP has been shown to be related to the erythrocyte's shape. ATP depleted cells are echinocytic but return to discocyte form upon restoration of normal ATP levels (11, 28, 31). Increasing internal calcium levels such as with the ionophore A23187 also produces echinocytes. This

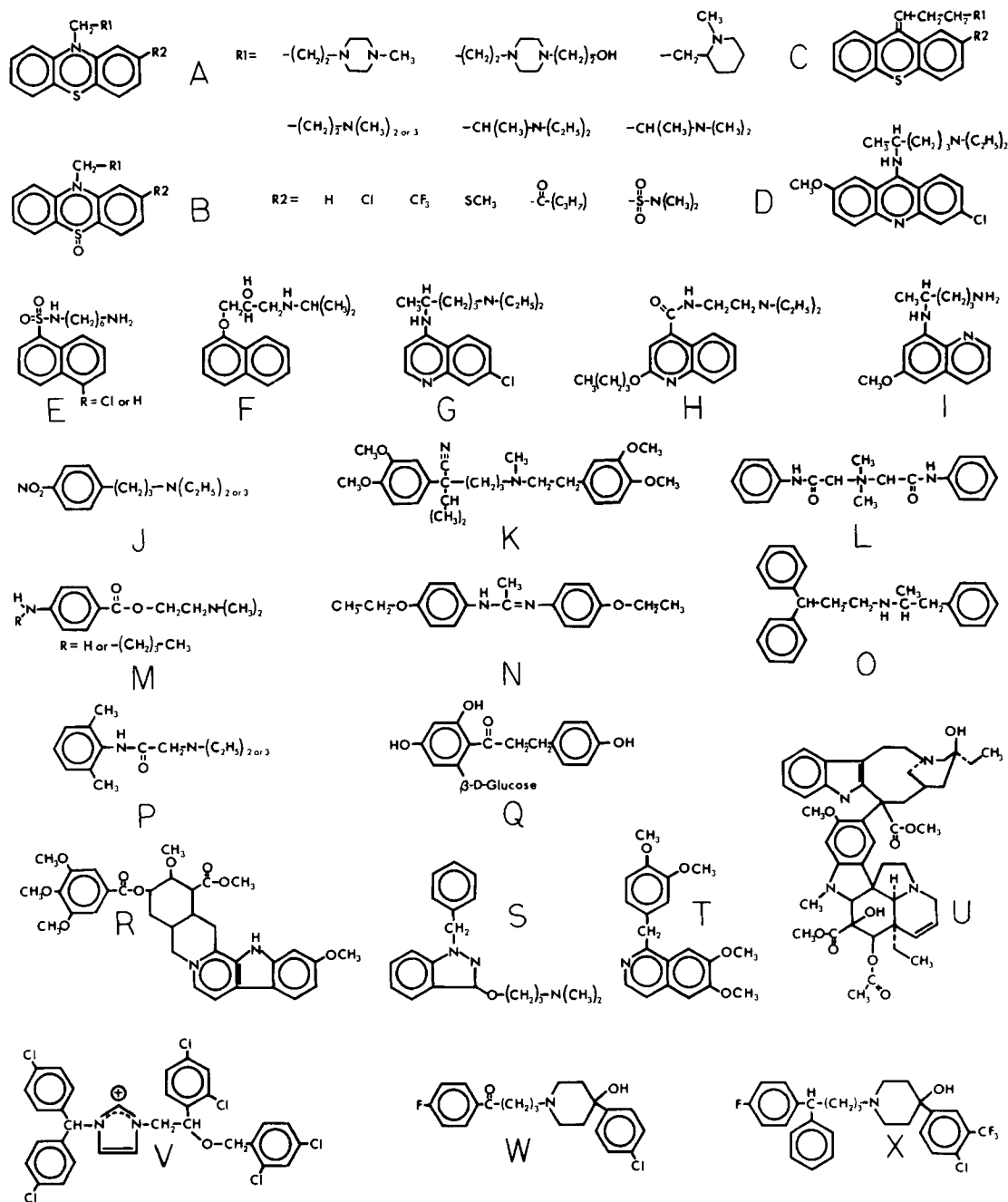


FIGURE 3 Drug structures. Summary of structures of cup forming and calmodulin inhibiting drugs whose activities are summarized in Table 1. (A) Phenothiazines. (B) Phenothiazine sulfoxides. (C) Thioxanthenes. R1 and R2 substitutions are for A–C. (D) Mepacrine equals Quinacrine. (E) Naphthalene sulfonamides W7 (R = Cl) and W5 (R = H). (F) Propranolol. (G) Chloroquine. (H) Dibucaine equals Cinchocaine. (I) Primaquine. (J) HK 27 (3° amine) and HK 25 (4° amine). (K) Verapamil. (L) QX 572. (M) Procaine (R = H) and Tetracaine (R = C<sub>4</sub>H<sub>9</sub>). (N) Phenacaine. (O) Prenylamine. (P) Lidocaine (3° amine) and QX 222 (4° amine). (Q) Phloridizin equals Phlorrhidzine. (R) Reserpine. (S) Benzydamine. (T) Papaverine. (U) Vinblastine. (V) R24571. (W) Haloperidol. (X) Penfluridol.

effect may be secondary to a depletion of cellular ATP which is hydrolysed by the membrane bound calcium ATPase pump (11). The ion pump binds and is regulated by calmodulin and influxes of calcium stimulate its activity (14, 25). Calmodulin inhibitors should reduce Ca<sup>++</sup> pump activity and thereby retard ATP depletion. This would favor cupped morphology. Furthermore, calmodulin inhibitors bind to calmodulin in its calcium loaded configuration and prevent Ca<sup>++</sup> release. This would have the effect of keeping free calcium levels low. ATP depletion and calcium influx also lead to two types of cross-

linking of cytoskeletal proteins into very high molecular weight complexes and should thereby stabilize the cell's morphology (27, 28, 41).

At least three routes of calmodulin regulation of cell shape are therefore possible in the erythrocyte: (a) binding of calmodulin to spectrin which alters its state of polymerization or association with cytoskeletal proteins; (b) modulation of spectrin phosphorylation via the calmodulin-dependent spectrin kinase; and (c) regulation of ionic environment and ATP levels via the calcium ATPase calcium pump or the ability of cal-

modulin itself to act as a calcium reservoir.

Calmodulin activity would be necessary for the stomatocyte to discocyte and discocyte to echinocyte transformations. Inhibitors of this activity would result in opposing morphological transformations due to restoring forces generated by the calmodulin "off" configuration of the cytoskeleton. This model can account for the activity of the known cup-forming drugs. However, the intercalation of drugs into the outer membrane leaflet may still be the appropriate explanation for the actions of many crenators. Brewer et al. (7) has previously drawn attention to the correlation between antihemolytic activity and CaM-inhibitory activity of several drugs. However, his earlier model (6) to explain how CaM might control erythrocyte shape is inconsistent with more recent findings. It was proposed that CaM and phosphorylation had antagonistic effects on the spectrin lattice which determined cell shape. It is now known (16) that CaM actually promotes spectrin phosphorylation via a CaM-dependent spectrin kinase, so their effects should be similar.

There are other examples of cell surface and shape regulation by calmodulin which establish a precedent for our model. Platelet shape and binding of Von Willebrand factor VIII to surface receptors are strongly affected by calmodulin inhibitors trifluoperazine and chlorpromazine, but not by their inactive sulfoxide derivatives (19). Surface antigen capping on lymphocytes is inhibited by local anesthetics and phenothiazines and calmodulin redistributes with the underlying cytoskeleton (24, 33, 34). In 3T3 and other cultured mammalian cells, local anesthetics, and phenothiazines alter the cell shape and cause a loss of microfilaments from the membrane (8, 31). Thus, regulation of cell shape at the level of membrane-cytoskeletal interaction may be a general role for calmodulin.

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