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Relationship between the Shape and the Membrane Potential of Human Red Blood Cells

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Summary. Microscopic observations of isotonic suspensions of human red blood cells demonstrate that cell shape is unaltered when the transmembrane electrical potential, or E_m, is set in the range −85 to +10 mV with valinomycin at varied external K^+, or K_o. E_m was measured with the fluorescent potentiometric indicator, diS-C_3(5), as calibrated by a ΔpH method. Repeating Glaser's experiments in which echinocytosis was attributed to hyperpolarization, we found that at low ionic strength the pH-dependent effects of amphotericin B appear to be unrelated to E_m. The effects of increased intracellular Ca^{2+}, or Ca_c, on echinocytosis and on E_m are separable. With Ca ionophore A23187 half-maximal echinocytosis occurs at greater Ca_o than that which induces the half-maximal hyperpolarization associated with Ca-induced K^+ conductance (Gardos effect). Thus, cells hyperpolarized by increased Ca_c remain discoidal when Ca is below the threshold for echinocytosis. With A23187 and higher Ca_o, extensive echinocytosis occurs in cells which are either hyperpolarized or at their resting potential. The Ca-activation curve for echinocytosis is left-shifted by low K_o, a new observation consistent with increased DIDS-sensitive uptake of ^{45}Ca by hyperpolarized cells. These results support the following conclusions: (1) the shape and membrane potential of human red blood cells are independent under the conditions studied; (2) in cells treated with A23187, the Gardos effect facilitates echinocytosis by increasing Ca_c.

Key Words red blood cells · membrane potential · calcium · Ca ionophore A23187 · fluorescence · cell shape

Introduction

A variety of chemicals or environmental conditions may transform the normal biconcave, discoidal shape of human red blood cells into cup-shaped forms, called stomatocytes, or into crenated, spiculed forms, known as echinocytes, with intermediate forms and many other variants also possible (Ponder, 1948; Deuticke, 1968; Bessis, 1973; Weed & Chailley, 1973). Although the association between increased intracellular Ca^{2+} and echinocytosis has been well-recognized (Weed, LaCelle & Merrill, 1969; Weed & Chailley, 1973; Dunn, 1974; Palek, Stewart & Lionetti, 1974; White, 1974; Sarkadi, Szasz & Gardos, 1976; Dreher et al., 1978, 1980), it is unknown whether the effect of Ca^{2+} on cell shape depends on a reaction of Ca^{2+} with membrane lipids, with membrane protein, or with cytoskeletal protein. It has recently been proposed (Glaser, 1979, 1982) that alterations of red cell shape are also correlated with changes in the transmembrane electrical potential, designated by E_m; specifically, that hyperpolarization is associated with echinocytosis and depolarization with stomatocytosis. Glaser's proposal was based on experiments in which human red cells were suspended in low ionic strength media at different pH's in the absence and presence of the channel-forming ionophores amphotericin B and nystatin, while the change in E_m was calculated by nonideal Gibbs-Donnan theory (*cf.* Freedman & Hoffman, 1979*a*). From measured Cl^- ratios, the resting potential of normal human red blood cells is −10 mV (Hastings et al., 1928), a value corresponding to an electric field strength across the membrane of the order of 10^4 V/cm. Glaser's hypothesis regarding E_m and cell shape, if generally valid, would be of interest because of the possible effect of such electrical forces on membrane structural components.

In this paper the relationship between E_m and red cell shape is re-evaluated by attempting to repeat Glaser's microscopic observations, by using isotonic cell suspensions in which E_m is varied by two other independent methods, and by measuring E_m with the fluorescent potentiometric indicator 3,3′-dipropylthiodicarbocyanine, or diS-C_3(5) (Hoffman & Laris, 1974; Sims et al., 1974). One method of varying E_m was to induce KCl diffusion potentials with the K^+ ionophore valinomycin at varied external K^+, designated by K_o. A second method utilized the Ca^{2+} ionophore A23187 in the presence of Ca_o (Reed, 1976). The resultant increase in intra-

cellular Ca^{2+}, or Ca_c, induces a selective increase in K^+ conductance, resulting in changes in E_m dependent on K_o. Increases in K^+ permeability attributed to Ca^{2+} were first described by Gardos (1959), and the associated changes in E_m dependent on Ca^{2+} have recently been characterized by the fluorescent potentiometric indicator technique (Freedman & Novak, 1983). While previous studies indicate that increased Ca_c is echinocytogenic at about 200 μM (Sarkadi et al., 1976), and that increased K^+ conductance is induced at μmolar or sub-μmolar levels of Ca_c (Blum & Hoffman, 1972), we sought to compare the Ca^{2+} activation curves for the change in E_m and for echinocytosis under equivalent conditions, and also to determine to what extent the Ca^{2+}-activation curve for the shape change might be affected by E_m in cells treated with A23187. These results have been partially summarized previously (Bifano & Freedman, 1982).

Materials and Methods

Preparation of Cells

Adult human blood was drawn by venipuncture into heparinized tubes and immediately centrifuged at 13,000 × *g* for 3 to 5 min at 4°C. The buffy coat was aspirated and the cells were washed three times by centrifugation and resuspension in chilled isotonic wash medium as described in the figure captions. The cells were then resuspended to 50% hematocrit (HCT) in the wash medium and kept on ice for use on the same day.

Microscopy

The cells were diluted to 2% HCT in buffered isotonic media (*see* figure legends) at desired K_o and Ca_o, and then observed microscopically before and at selected time intervals after addition of 1 μM valinomycin (Sigma Chemical Co., St. Louis, Mo.), 0.1 μM gramicidin D (Sigma), or 0.8 μM Ca ionophore A23187 (Calbiochem-Behring Corp., LaJolla, Calif.).

In experiments with the channel-forming ionophore, amphotericin B, the experimental method was similar to that described by Glaser (1979, 1982). For this set of experiments the final wash of cells utilized media containing (mM): 30 NaCl, 224 sucrose, 0.1 EDTA, and 5 HEPES, pH 7.4 or 5.1 (296 mOsm). Cell suspensions were then incubated for 1 hr at 25°C in media containing varied amounts of *amphotericin B* (Fungizone, Squibb, Princeton, N.J.; 45% amphotericin B, 37% Na deoxycholate as solubilizer, and 18% Na phosphate buffer) prior to microscopic observations.

Stock solutions of valinomycin (3 mM), gramicidin D (0.1 mM), and A23187 (2.4 mM) in EtOH were stored at −20°C. Stock solutions of amphotericin B (5 mg/ml) in distilled water were made on the day of use. Note that amphotericin B concentrations given in the text refer to Fungizone, only 45% of which is amphotericin B.

For microscopy 25 μl of the 2% HCT red cell suspensions were placed on carefully washed vinyl plastic slides (A.H. Thomas, Philadelphia, Pa.) and a plastic coverslip was applied. While 2% HCT yielded the best photomicrographs, controls between 0.17 and 3.0% HCT under the same experimental conditions gave the same % nondiscs. The slides and coverslips were handled only with forceps after having been washed in detergent, followed by extensive rinsing. Bovine serum albumin (0.01%) was included in all media in order to avoid the crenation which occurred to some extent between slide and coverslip without albumin despite the use of thoroughly cleaned plastic (*cf.* Ponder, 1948). The use of glass slides which were dipped in EtOH and flamed also did not totally and consistently prevent the discsphere transformation (*cf.* Trotter, 1956), even when a filter paper spacer separated slide and coverslip. Bovine serum albumin at 0.01% was found to be the minimal amount that prevents artifactual shape changes while avoiding stomatocytosis, which may reach up to 50% at 0.1% albumin (Jay, 1975). The cells were viewed and photographed using a Zeiss research microscope equipped with Nomarski interference contrast optics, a 40× objective with 5 or 10× oculars, and a Cs-Matic camera for 35 mm film or a Polaroid camera with type 665 P/N or type 084 film. Changes in cell shape were quantitated by estimating the percentage of cells, from a total count of at least 200, that were neither discocytes nor stomatocytes ("% nondiscs" or "% echinocytes"). All cells in any stage of the transformation from discocyte to echinocytes I, II and III, to spheroechinocyte and to spherocyte were included in the percentage of nondiscoid cells. In describing the various red cell forms encountered in our experiments, the nomenclature of Bessis (1973) was found to be extremely useful.

Membrane Potentials, E_m

E_m was estimated from the fluorescence of the permeant cyanine dye, 3,3′-dipropylthiodicarbocyanine iodide, or diS-C_3(5), using the technique introduced by Hoffman and Laris (1974). The dye fluorescence was calibrated for mV by a ΔpH method (*see* Results, and Freedman and Novak, 1983), a procedure which utilized DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene from Sigma, freshly prepared at 10 mM in 20 mM NaOH), valinomycin, and the proton ionophore FCCP (carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone, gift of Dr. P.G. Heytler, E.I. Dupont De Nemours and Co.). The necessity of including albumin in the suspensions for microscopy interfered with the monitoring of E_m by WW781 but not by diS-C_3(5). For studies with Ca^{2+}, controls in the absence of albumin showed that the inhibition of the Gardos effect by diS-C_3(5) (Simons, 1976*a*, 1979) decreased the size of the Ca^{2+}-induced initial change in fluorescence; however, the concentration of Ca_o giving half-maximal activation of the change in E_m was comparable with diS-C_3(5) and the noninhibitory oxonol dye, WW781 (*see* Results, and Freedman and Novak, 1983). All fluorescence changes were measured with an SLM 8000S photon counting fluorimeter as described previously (Freedman & Novak, 1983). DiS-C_3(5) was a gift from Dr. A. Waggoner, Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pa.

Ca Influx

Uptake of Ca^{2+} by red cells was determined using ^{45}Ca as tracer. Fresh blood was drawn and washed as described above in chilled medium containing (mM): 145 NaCl, 5 KCl and 5 HEPES (pH 7.4 at 25°C). The flux media contained (mM): 0.7 $CaCl_2$ with 0.2 μCi/

ml ^{45}Ca (20 mCi/mg, ICN, Irvine, Calif.), 0.5 EGTA, 5 HEPES (pH 7.4 at 25°C) and either 1 KCl and 140 NaCl or 90 KCl and 60 NaCl, and was equilibrated in a shaking water bath at 25°C. The red cells were added to 3% HCT 5 min before the addition at zero time of 0.8 μM A23187. Prior to the addition of A23187, and at 5, 10 and 20 min thereafter, three 10-ml samples were transferred to three 40-ml glass centrifuge tubes (Pyrex) that were kept on ice before and during sampling. Three cold finger condensers (glass, 16.5 × 2.2 cm), connected in series to a circulating water bath set at 1.5°C, were then placed for at least 1 min in each of the ice-cold centrifuge tubes, thus cooling the suspensions to 1.5°C within 1 min. Controls showed that A23187-mediated ^{45}Ca influx was inhibited by about 90% at 1.5°C. Each ice-cold 10-ml aliquot was then transferred and centrifuged using polypropylene microcentrifuge tubes (Eppendorf, 0.4 ml capacity) with attached syringe barrels as described previously (Freedman & Hoffman, 1979*a*).

After separation of cells and supernatant, the packed cells (about 0.1 ml) were hemolyzed in 1.3 ml distilled water. Aliquots (0.1 ml) of each hemolysate were added to 4.9 ml of Drabkin's reagent for measurement of hemoglobin ($[Hb]_L$ (g Hb/ml), at 540 nm in comparison with cyanmethemoglobin standards (Hycel, Inc., Houston, Tex.). Another aliquot (1 ml) of each hemolysate was added to 1 ml of cold 10% trichloroacetic acid (TCA), and after mixing and centrifugation, 1 ml of each clear supernatant was added to 10 ml of aqueous counting scintillant (ACS, Amersham Corp., Arlington Heights, Ill.) and counted, CPM_c, in a scintillation counter (LS7500 Beckman Inst., Palo Alto, Calif.). The Ca^{2+} uptake prior to the addition of ionophore was subtracted from the uptake for each time point after the addition of ionophore in order to correct for trapped extracellular fluid and nonspecific binding. Aliquots (0.1 ml) of the supernatants from each cell suspension were added to 1 ml of distilled water plus 1 ml of 10% TCA, and after mixing and centrifugation 1 ml was then added to 10 ml ACS and counted, $(CPM)_o$. The formula for computing Ca_c (μmol/g Hb) was as follows:

$$Ca_c = (2\ CPM_c[Ca]_o)/(21\ CPM_o[Hb]_L)$$

where 21 is the dilution factor for the supernatants, and 2 is the dilution factor for the cell extracts.

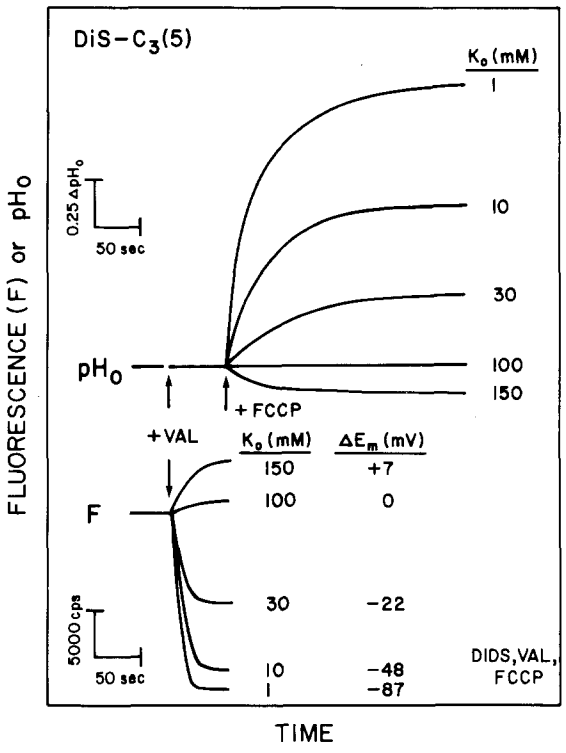

Fig. 1. Calibration of diS-$C_3(5)$ by the ΔpH method. After washing three times in unbuffered 150 mM NaCl, the cells were resuspended to 25% HCT and incubated for 10 min at 25°C with freshly prepared 10 μM DIDS, and then kept on ice until needed. Cuvettes contained 2.5 ml of media consisting of x mM KCl, where x = 0, 0.1, 0.3, 1.0, 3.0, 10, 30, 100 or 150, and (150 − x) mM NaCl. DIDS-treated cells were added to 0.42% HCT, followed by 0.83 μl of diS-$C_3(5)$ (0.2 mg/ml stock in EtOH). After equilibration to 23°C and adjustment of the initial pH to 7.40 with 0.1 M NaOH, the diffusion potential was induced with 2 μM valinomycin (VAL) while the dye fluorescence (F) and pH_o were recorded simultaneously. After F reached a new plateau, 5 μM FCCP was added and the change in pH_o was measured. The values shown for ΔE_m (mV) were computed from Eq. (1) as described in the text

Results

Measurement of E_m by diS-$C_3(5)$ Fluorescence

Changes in the fluorescence of diS-$C_3(5)$ have been related to mV values in the past by using the constant field theory to calculate diffusion potentials (Hoffman & Laris, 1974; *see* Cohen & Hoffman, 1982), by dye binding measurements (Hladky & Rink, 1976; Tsien & Hladky, 1978), and by Gibbs-Donnan equilibrium potentials (Freedman & Hoffman, 1979*b*; *see* Freedman & Laris, 1981). A new calibration method utilizing simultaneous measurements of fluorescence and pH_o in unbuffered suspensions of cells treated successively with DIDS, valinomycin and the proton ionophore FCCP was recently devised in conjunction with a study of the effect of Ca^{2+} on red cell E_m, as determined with a rapidly responding, presumably impermeant oxonol dye, WW781 (Freedman & Novak, 1983). The results in Figs. 1 and 2 show for diS-$C_3(5)$ how the ΔpH calibration method measures KCl diffusion potentials induced by valinomycin at varied K_o. The washed cells are treated with 10 μM DIDS in order to prevent the Cl^-/HCO_3^- exchanger from affecting the proton distribution (Wieth, Brahm & Funder, 1980; *cf.* Macey, Adorante & Orme, 1978). The lower set of traces in Fig. 1 shows the fluorescence changes after addition of 1 μM valinomycin to isotonic suspensions with K_o ranging from 1 to 150 mM. The upper set of traces in Fig. 1 shows that valinomycin imparts a negligible ΔpH_o at any K_o because of the low proton permeability of DIDS-treated

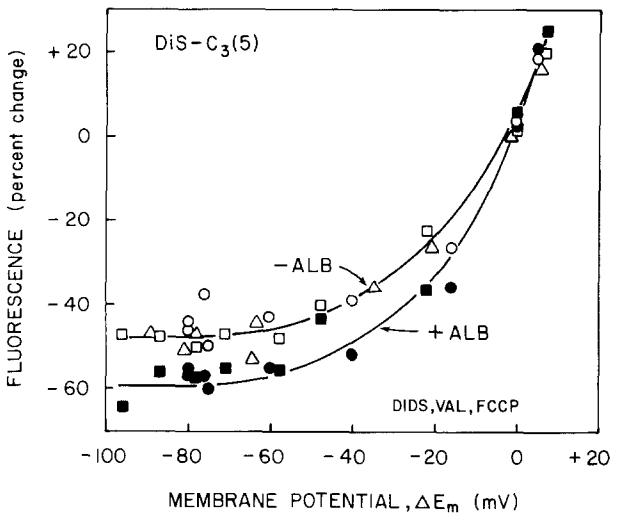

Fig. 2. Calibration of the change in fluorescence of diS-$C_3(5)$ versus the change in membrane potential, ΔE_m, obtained by the ΔpH method in the absence and presence of albumin (ALB, 1 mg/ml). The experimental protocol was the same as for Fig. 1. ΔE_m was computed from Eq. (1) (*see text*) and was assumed to be independent of the presence of 0.1% albumin (*see text*). The circles, squares and triangles represent data from three independent experiments, with representative traces of fluorescence and pH_o shown in Fig. 1

cells. Assuming that subsequent addition of 5 μM FCCP allows protons to equilibrate with E_m, the resulting changes in pH_o are related to ΔE_m as follows:

$$\Delta E_m = -(2.303\ RT/F)\Delta pH_o, \quad (1)$$

where 2.303 RT/F = 58.7 mV at 23°C. Typical values for ΔE_m calculated from Eq. (1) are shown on Fig. 1 beside the fluorescence traces. The records of fluorescence after the addition of FCCP are not shown because the proton ionophore quenches the fluorescence of diS-$C_3(5)$.

Resultant plots of percentage change in fluorescence versus ΔE_m are shown in Fig. 2 for three independent experiments (open circles, squares and triangles). Linear regression of the data between −25 and +7 mV yields a relationship between the change in fluorescence and ΔE_m of +1.6% ΔF/mV. This value obtained by the ΔpH method for DIDS-treated cells corresponds well with the value of +1.7% ΔF/mV previously reported from Gibbs-Donnan potentials for cells unexposed to DIDS, and is also equal to the value of +1.6% ΔF/mV estimated for cells treated with valinomycin and assuming that P_K/P_{Cl} = 20 (Freedman & Hoffman, 1979*b*). Direct comparisons with the dye binding studies of Hladky and Rink (1976) and of Tsien and Hladky (1978) are not possible because of the different dye concentrations and hematocrits employed, and because of differences in the way their data were normalized and reported.

In two experiments the changes in fluorescence and pH_o induced by valinomycin at varied K_o were measured in the presence and absence of 0.1% albumin (Fig. 2, solid circles and squares). The effect of albumin on dye calibration was tested because it was present at 0.01% in the media used for the studies of cell shape (*see* Materials and Methods), and also because of a recent suggestion based on studies with neutrophils that it might increase the range of linearity of the calibration curve of diS-$C_3(5)$ (Simchowitz, Spilberg & deWeer, 1982). Because the buffering capacity of albumin precludes the use of Eq. (1), and because it seems reasonable to assume that low concentrations of albumin do not affect ΔE_m, the calibration curve in the presence of albumin is based on the fluorescence changes obtained in the presence of the protein and on the values of ΔE_m calculated from ΔpH_o obtained in the absence of albumin. The results show that while albumin promotes slightly larger fluorescence changes, the range of linearity is unaltered by the protein in suspensions of human red cells. The nonlinear relationship between $\%\Delta F$ and ΔE_m arises from the redistribution mechanism by which diS-$C_3(5)$ monitors ΔE_m (Sims et al., 1974; Hladky & Rink, 1976; Tsien & Hladky, 1978). Saturation of the response of diS-$C_3(5)$ implies that precise values for hyperpolarization are available to about −60 mV. By the ΔpH calibration procedure, the changes in ΔE_m have been quantitated, permitting an evaluation of the relationship between E_m and cell shape as described below.

Cell Shape and Valinomycin-Induced KCl Diffusion Potentials

In order to detect a possible effect of E_m on the shape of human red blood cells, valinomycin was added to isotonic red cell suspensions at varied K_o and the cells were photographed before and at selected times after the addition of the K^+ ionophore. The K_o of the suspensions were 0 (nominally) or 1, 90 and 150 mM, chosen so that addition of valinomycin resulted in hyperpolarization, no change in E_m (designated as the null point), and depolarization, respectively. Typical photomicrographs of DIDS-treated cells, along with the values of the diffusion potential measured on the same cells, are shown in Fig. 3. Before valinomycin DIDS itself caused the formation of 20 to 35% stage I echinocytes, defined as discs with irregularly contoured surfaces but without spicules (Fig. 3*A*, top, *pre*). At

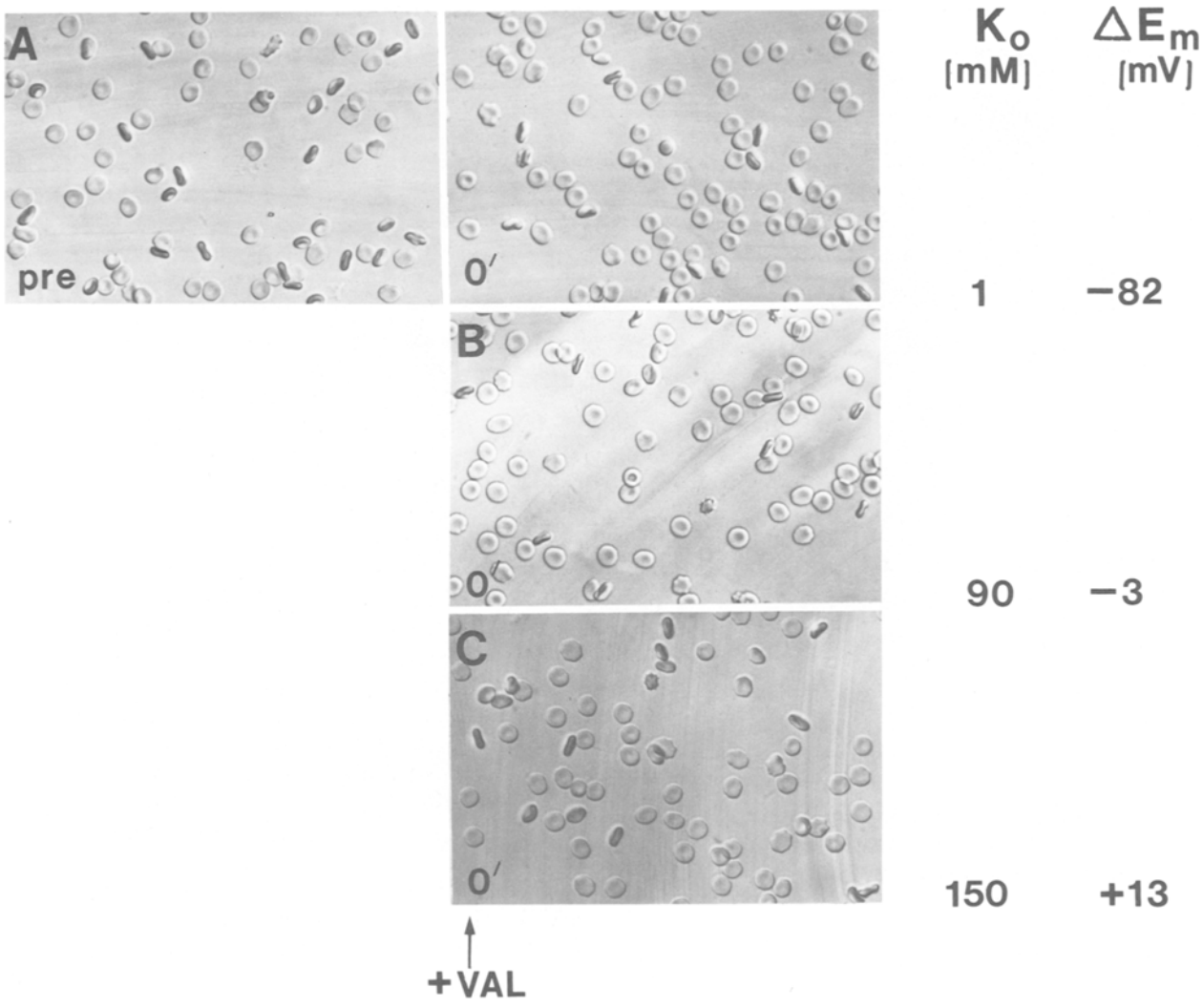

Fig. 3. The shape and diffusion potentials of DIDS-treated human red blood cells before and after the addition of valinomycin at varied K_o (Nomarski interference contrast). After washing three times in unbuffered 150 mM NaCl, the cells were adjusted to 50% HCT and kept on ice. The cells were incubated at 0.4% HCT for 30 min with 1 μM DIDS, and then transferred to cuvettes containing 2.5 ml of media consisting of x mM KCl and (150 − x) mM NaCl where x = 1, 90 or 150. Then 25 μl of 1% albumin was added, followed by 8.3 μl of diS-$C_3(5)$ (0.2 mg/ml stock). Fluorescence and pH_o were recorded simultaneously before and after addition of 2 μM valinomycin and 1 μM FCCP. Diffusion potentials were computed from Eq. (1) (*see text*). Aliquots (25 μl) for photomicroscopy were removed from the cuvettes before and immediately after addition of valinomycin

each K_o, the percentage of cells with spicules immediately after addition of valinomycin remained less than 10% while the diffusion potentials ranged from −82 to +13 mV (Fig. 3*A–C,0′*).

Representative photomicrographs of cells unexposed to DIDS, where the range of ΔE_m is somewhat less than with DIDS, are shown in Fig. 4. At low K_o, there was no change in cell shape immediately or at 5 min after the addition of valinomycin (Fig. 4*A*, top, 0′ and 5′). At 15 min the cells appeared flattened and shrunken—a shape denoted as "leptocyte" (Bessis, 1973; Dreher et al., 1980). The shrunken appearance at 15 min (Fig. 4*A*, 15′) is due to cellular dehydration from loss of KCl rather than to the effect of hyperpolarization, as the change in fluorescence of diS-$C_3(5)$ after the addition of valinomycin was immediate. Addition of valinomycin at 90 mM K_o, near the null point, resulted in no observable change in cell shape over 15 min (Fig. 4*B*, middle row). Depolarization by addition of valinomycin at 150 mM K_o did not change cell shape either (Fig. 4*C*, bottom row). Therefore, under the isotonic conditions in these experiments (which were replicated with five different donors without DIDS and twice with DIDS) no effect of E_m on red cell shape was detected. Identical results were ob-

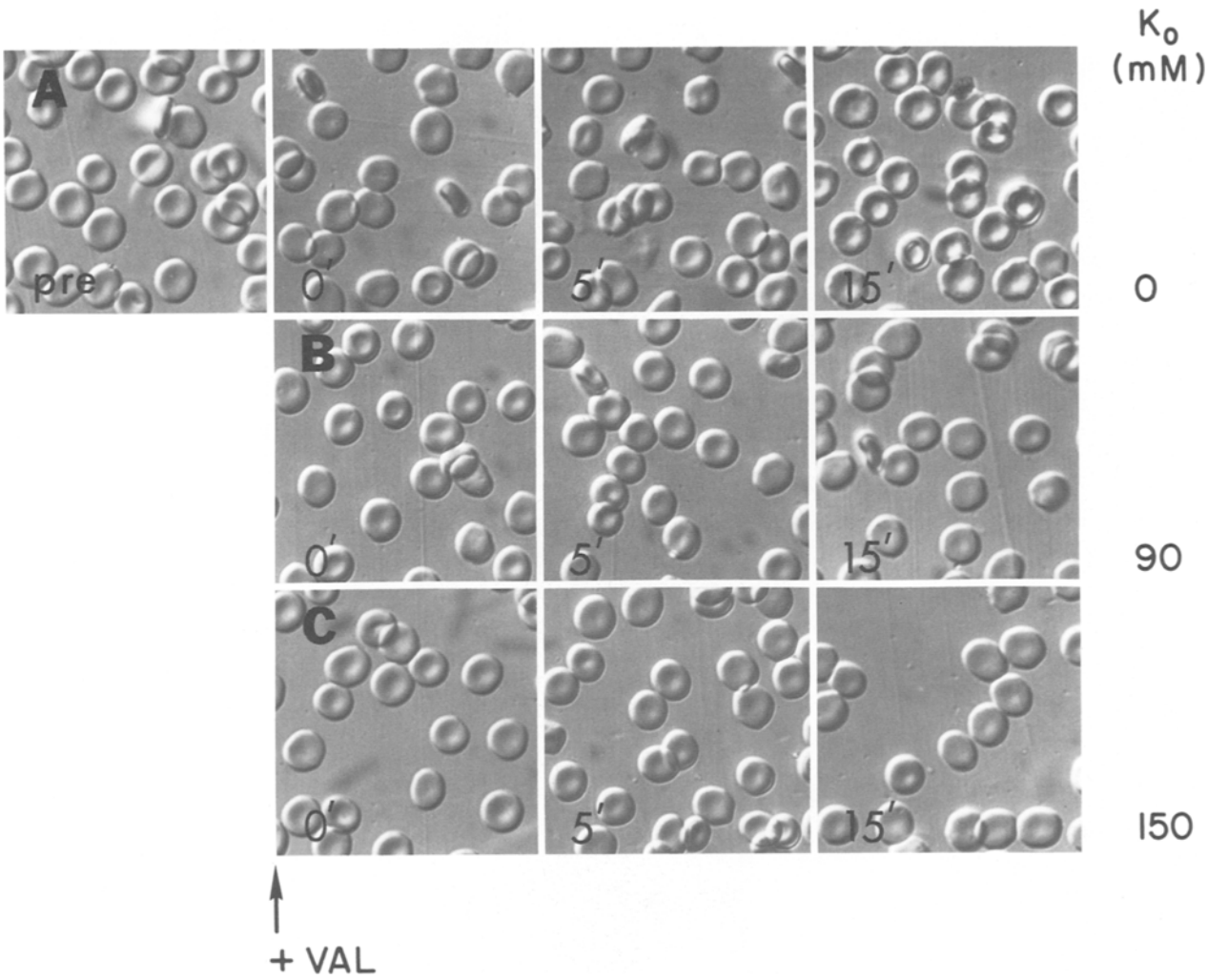

Fig. 4. The shape of human red blood cells before and after the addition of valinomycin at varied K_o (Nomarski interference contrast). After washing the cells in chilled media containing 150 mM NaCl, 5 mM HEPES (pH 7.35 at 25°C), 60 μl aliquots of 50% HCT cell suspension were added to 3 ml of media containing (150 − x) mM NaCl, 5 mM HEPES (pH 7.35 at 25°C), and x mM KCl, where x equals 0 (top panel), 90 (middle panel) or 150 (lower panel), followed by addition of 30 μl of 1.0% bovine serum albumin. Aliquots of these suspensions were photographed before, and immediately (0 min) after, and at 5 and 10 min after the addition of 10 μl of 300 μM valinomycin (in EtOH, 1 μM final concentration). (*A*): Cells hyperpolarized. (*B*): Cells at null point. (*C*): Cells depolarized

tained with 0.1 μM gramicidin D at 1 and 90 mM K_o in media in which the NaCl was replaced with choline Cl, thus giving a range of E_m comparable to that with valinomycin (*not shown*).

Cell Shape at Reduced Ionic Strength with Amphotericin B or Valinomycin

Since the results with isotonic suspensions indicated that the shape of red cells treated with valinomycin (Figs. 3 and 4) or gramicidin (*not shown*) is independent of E_m, the effects of amphotericin B at low ionic strength were re-examined, using conditions similar to those described by Glaser (1982). Cells were washed and incubated in isotonic low ionic strength media containing sucrose and 30 mM NaCl (*see* Materials and Methods) at pH 5.1 or 7.4, for which Glaser (1982) computed E_m to be +46 and +30 mV, respectively. The positive potentials are due to reversal of the Cl^- gradient. Without amphotericin B (Fig. 5, top row) there was some increase in cupping (stomatocyte I), which, though difficult to quantitate, was qualitatively in agreement with Glaser (1982). The effect on cell shape of the cation-selective ionophore amphotericin B was tested between 0 and 1 mg/ml (Fig. 5). At pH 5.1, below the isoelectric point of Hb, E_m remains positive when

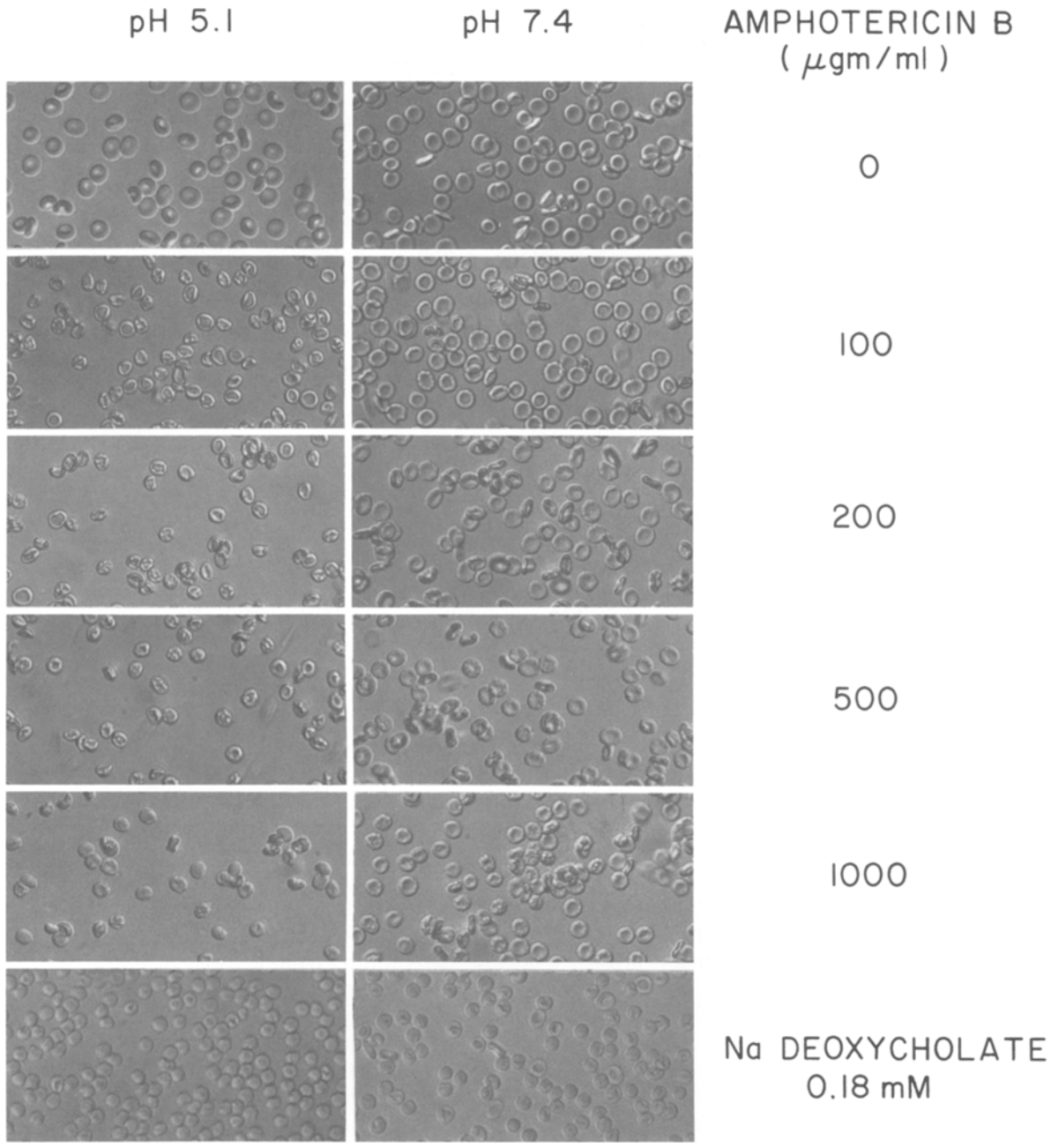

Fig. 5. The shape of human red blood cells at low ionic strength with amphotericin B. After washing the cells twice in media containing 145 mM NaCl, 5 mM KCl, and 5 mM HEPES buffer (pH 7.4 at 25°C), the suspension was divided in half and washed again in low ionic strength media (*see* Materials and Methods) at pH 5.1 or 7.4 as indicated. Then 0.01% albumin and varied amounts of Amphotericin B were added and the suspensions were incubated at 2% hematocrit for 1 hr at 25°C, followed by photomicroscopy. The results shown above are typical of two experiments at varied drug concentration and of 4 others at 100 μg/ml. The bottom row is a control without amphotericin B, but including 0.18 mM Na deoxycholate (*see text*)

amphotericin B increases cation permeability and allows Donnan equilibrium of Na^+ and K^+. After 1 hr at 100 μg/ml drug (pH 5.1), the cells were uniformly and extensively cupped with virtually no echinocytosis (Fig. 5), also in agreement with Glaser (1982). At 200 to 1000 μg/ml amphotericin B (pH 5.1), the cells developed blunt spicules and resembled stomato I-acanthocytes. Upon incubation at pH 7.4, above the isoelectric point of Hb, addition of amphotericin B results in a Donnan potential of

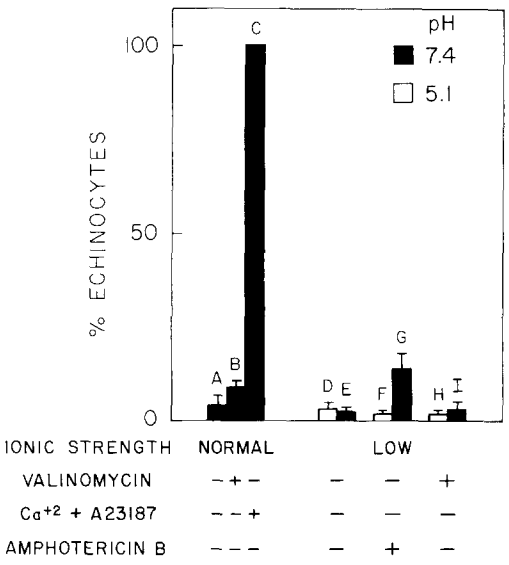

Fig. 6. Percentage echinocytes ("nondiscs") with valinomycin, Ca^{2+} plus A23187, and amphotericin B. Cell suspensions were prepared at normal ionic strength with 1 mM K_o and the percentage echinocytes determined without ionophores (bar *A*), 5 min after addition of 1 μM valinomycin (bar *B*), and 15 min after addition of 0.8 μM A23187 with 5 mM Ca_o (bar *C*), as described in Figs. 4 and 7 and in Materials and Methods. Cells were also incubated for 1 hr at low ionic strength at pH 5.1 (clear bars) and at pH 7.4 (shaded bars) in the absence (bars *D* and *E*) and presence of 100 μg/ml amphotericin B (bars *F* and *G*), and for 5 min with 1 μM valinomycin (bars *H* and *I*). Error bars indicate 1 SD for experiments with blood from 3 to 4 donors

−20 mV. Whereas Glaser (1982) reported a uniform population of echinocytes, we observed primarily stage I echinocytes at 100 to 500 μg/ml amphotericin B (Fig. 5), with increased cell aggregation at 1000 μg/ml (pH 7.4). In 4 similar experiments at 100 μg/ml (pH 7.4) we estimated 14 ± 4% (SD, $n = 4$) echinocytes (Fig. 6, bar *G*), but it was difficult to distinguish between discs and stage I echinocytes. Controls at pH 5.1 and 7.4 incubated at low ionic strength without amphotericin B (Fig. 5, top row, and Fig. 6, bars *D* and *E*) showed less than 5% echinocytes. Since the pharmaceutical preparation of amphotericin B used both by Glaser and by us contains 37% Na-deoxycholate to solubilize the ionophore, we also tested the effect of the detergent alone on cell shape using the same media as in Fig. 5. At both pH 5.1 and 7.4 with 0.18 and 0.9 mM Na-deoxycholate corresponding to the concentrations present with 200 and 1000 μg/ml amphotericin B, cell morphology was uniformly altered to spherostomatocytes (Fig. 5, bottom row), an effect which certainly confounds the interpretation of Glaser's observations. Quenching of diS-$C_3(5)$ fluorescence by amphotericin B precluded the measurement of E_m.

An independent test of the possible relationship between hyperpolarization and echinocytosis at low ionic strength is to add valinomycin at low K_o. At both pH 7.4 and 5.1 using the same media as for experiments with amphotericin B, addition of valinomycin resulted in flattened cells with smooth rims after 15 min, as expected from KCl loss and dehydration (*not shown*). Echinocytosis was less than 5% at low ionic strength (Fig. 6, bars *H* and *I*) and less than 10% at normal ionic strength (Fig. 6, bars *A* and *B*). In the low ionic strength suspensions, addition of valinomycin at 1 mM K_o decreased the fluorescence of diS-$C_3(5)$ by an extent comparable to that obtained upon hyperpolarization at normal ionic strength. These observations (Fig. 6) suggest that the effects reported by Glaser (1979, 1982) with amphotericin B may be due to a drug membrane interaction which is dependent on pH but unrelated to E_m.

Cell Shape and Ca-Induced KCl Diffusion Potentials

As an alternate means of varying E_m at normal ionic strength, KCl diffusion potentials were also induced with the calcium ionophore A23187 in the presence of Ca_o (Freedman & Novak, 1983). Addition of 0.8 μM A23187 at 5 mM Ca_o, 0.5 mM EGTA and with K_o at either 1 or 90 mM resulted in hyperpolarization or no change in E_m, respectively, as determined with diS-$C_3(5)$. At both 1 and 90 mM K_o, the cells changed from discocytes to echinocytes and then to spherocytes within 15 min (Fig. 7, *A* and *B*, top and middle rows). Thus, Ca-induced echinocytosis appears to be independent of E_m, as it occurs at both low and null point K_o, and also is not dependent on cellular dehydration from net loss of KCl (Fig. 7*B*, middle row). Dreher et al. (1978) studied fixed cells by scanning electron microscopy and reported that echinocytosis after addition of A23187 in the presence of Ca_o was prevented when K^+ was the major external cation, in contrast to Fig. 6, middle row. However, subsequent clarification indicated that high K^+ buffer prevented the Ca-induced change in cell rigidity while still allowing the shape change (Dreher et al., 1980), in agreement with Fig. 7, middle row.

At a total Ca_o of 0.5 mM and with 0.5 mM EGTA, addition of A23187 at 1 mM K_o results in maximal hyperpolarization, as determined with diS-$C_3(5)$ (Fig. 8, broken line), but no echinocytosis was noted within 15 min (Fig. 6*C*, bottom row). Thus, there is a Ca_o at which the Ca-induced change in K^+ permeability and the resultant hyperpolarization of the cells at low K_o occurs, but at which the discoid shape is retained.

In order to compare the Ca_o at which the changes in K^+ conductance and cell shape are acti-

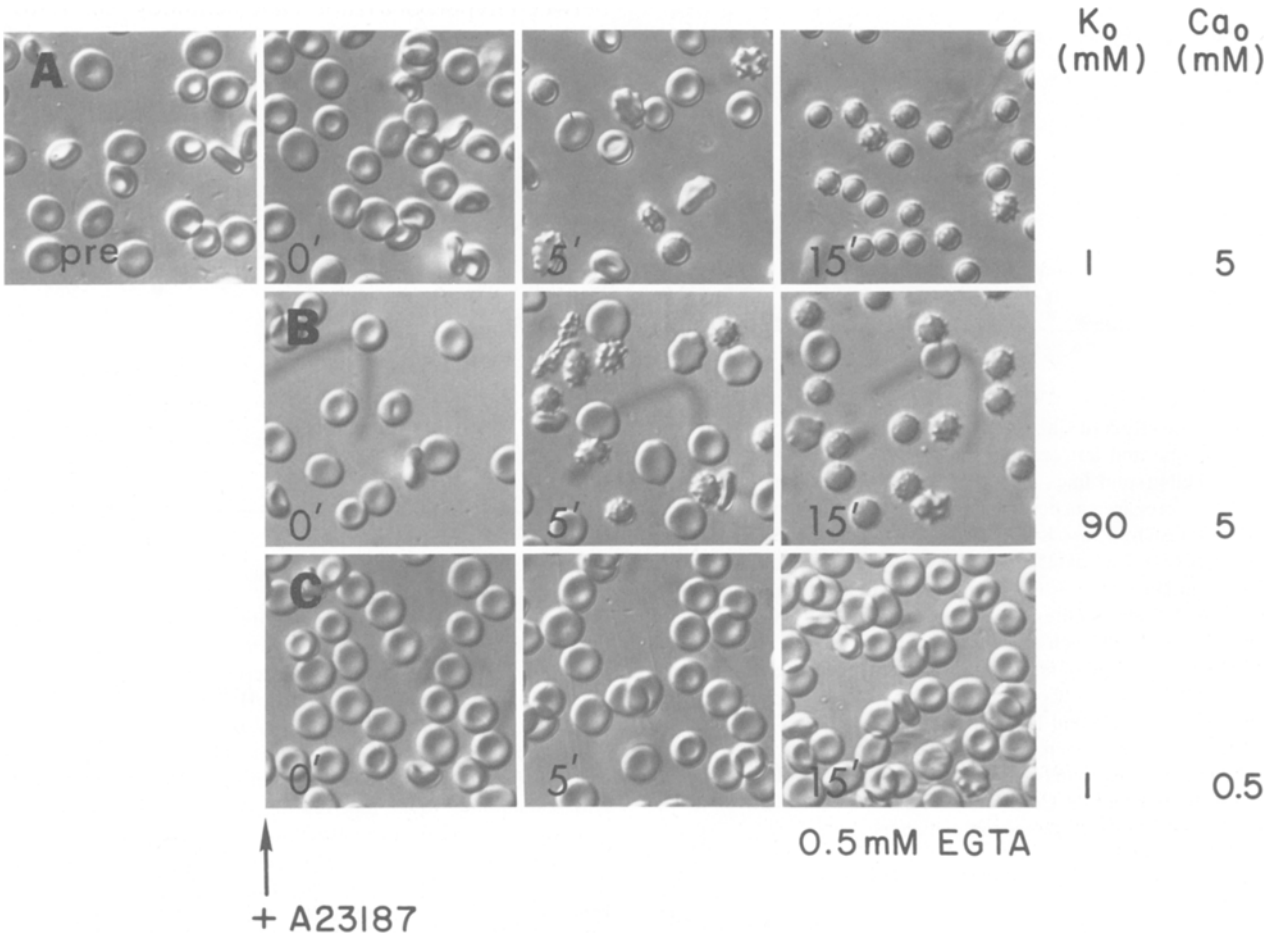

Fig. 7. The shape of human red blood cells in the presence of Ca_o before and after the addition of Ca ionophore A23187 at varied K_o and Ca_o (Nomarski optics). Cell suspensions (3 ml) were prepared as described in Fig. 4 in media containing 5 mM HEPES (pH 7.35 at 25°C), 0.5 mM EGTA, 0.01% bovine serum albumin and the following: (*A*): 5 mM $CaCl_2$, 1 mM KCl and 141.5 mM NaCl; (*B*): 5 mM $CaCl_2$, 1 mM KCl and 149 mM NaCl; (*C*): 0.5 mM $CaCl_2$, 1 mM KCl and 149 mM NaCl. These suspensions were then photographed before, and immediately (0 min) after, and at 5 and 15 min after the addition of 1 μl of 2.4 mM A23187 (in EtOH, 0.8 μM final concentration). (*A*): Cells hyperpolarized. (*B*): Cells at null point. (*C*): Cells hyperpolarized

vated, dose response curves for each effect under equivalent conditions were determined (Fig. 8). At 1 mM K_o, the change in fluorescence of diS-C_3(5) after the addition of A23187 is half-maximal at a total Ca_o of 350 μM. From the EGTA binding constants (Raaflaub, 1956; Simons, 1976*b*), the half-maximally effective ionized external Ca^{2+} is approximately 0.1 μM, in satisfactory agreement with the value previously obtained with the oxonol dye, WW781 (Freedman & Novak, 1983). In comparison (also at 1 mM K_o), 50% of the cells were nondiscs at 15 min at 640 μM total Ca_o, with maximal shape transformation at 800 μM total Ca_o. Allowing for 500 μM EGTA, the free external Ca^{2+} required for the shape change is 300 μM, in satisfactory agreement with the previously reported minimal concentration of 200 μM Ca required to transform discocytes to a homogeneous population of spherocytes (Sarkadi et al., 1976). These results confirm that Ca-induced K conductance and echinocytosis are independent processes since they have widely different thresholds for activation by Ca^{2+}.

In order to ascertain whether Ca-induced echinocytosis might be affected by E_m, dose response curves for the effect of Ca_o on the shape change at 90 mM K_o were also determined (Fig. 8). The free external Ca^{2+} that resulted in half-maximal transformation of the cells at 90 mM K_o was substantially higher at 320 μM than the 140 μM free Ca^{2+} required at 1 mM K_o. The Ca-activation curves for the shape change are situated such that at 0.7 mM total Ca_o, lowering K_o from 90 to 1 mM should result in increased echinocytosis. Accordingly, experiments were performed in which A23187 was added to cells suspended at 90 mM KCl, 60 mM NaCl, 0.7 mM CaCl, 0.5 mM EGTA, 5 mM HEPES

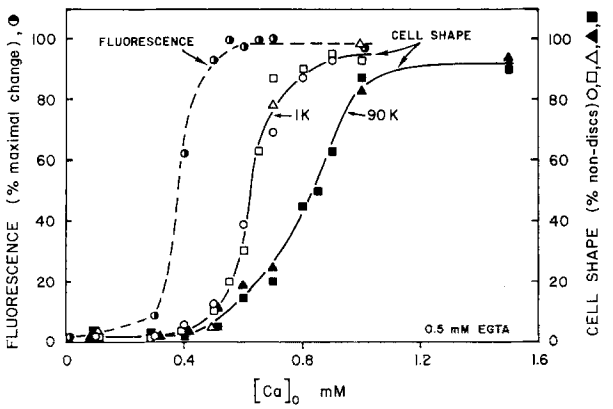

Fig. 8. The effect of Ca^{2+} on the change in diS-C_3(5) fluorescence (dotted line and left ordinate) and on the shape of human red blood cells (solid lines and right ordinate). Cell suspensions (3 ml) were prepared as described in Fig. 4 in media containing 0 to 1.5 mM $CaCl_2$, x mM KCl (x = 1 or 90), 0.5 mM EGTA, 5 mM HEPES (pH 7.35 at 25°C), 0.01% bovine serum albumin, and sufficient NaCl (150 − x − 3/2 Ca) so that each solution was 300 imOsm. Fifteen minutes after the addition of 0.8 μM A23187, at least 200 cells of each suspension were observed and counted and the percentage of nondiscoid forms was recorded (*see* Materials and Methods). Controls showed that the % nondiscs increased between 5 and 15 min (e.g. Fig. 7, top row). Also shown (dotted line) are the percentage changes of fluorescence, $\%\Delta F$, of the optical potentiometric indicator, diS-C_3(5), as determined at 1 mM K_o and varied Ca_o under conditions equivalent to those used to observe the changes in cell shape

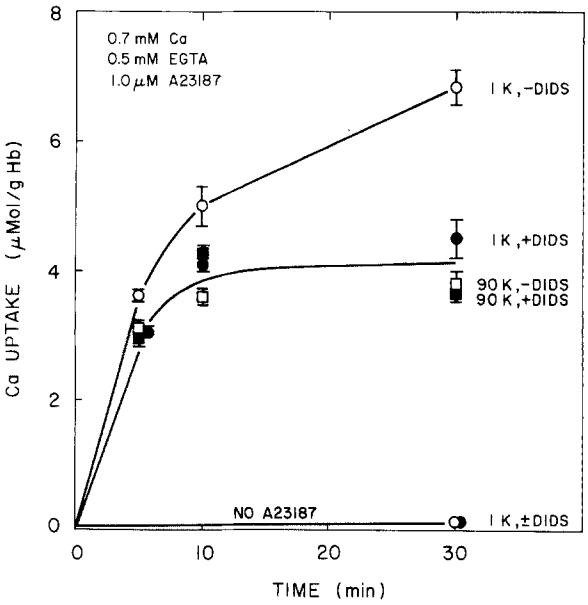

Fig. 9. The effect of DIDS on calcium uptake at 1 and 90 mM K_o into human red blood cells treated with A23187. Cell suspensions were prepared as described in Materials and Methods in flux media containing 5 mM HEPES (pH 7.35 at 25°C), 0.5 mM EGTA, 0.01% bovine serum albumin, 0.7 mM $CaCl_2$, 0.2 μCi/ml $^{45}CaCl_2$ and either 90 mM KCl and 60 mM NaCl or 1 mM KCl and 149 mM NaCl. Aliquots were taken before and at 5, 10 and 30 min after the addition of 0.8 μM A23187. The cells and supernatants were then separated and analyzed for beta activity (*see* Materials and Methods). The squares (90K) and circles (1K) represent the mean of three samples for each time point and the error bars represent two standard deviations. Empty symbols are for cells unexposed to DIDS, while filled symbols represent cells pretreated at 25% HCT with 10 μM DIDS at 25°C for 30 min and then centrifuged and kept on ice until used for the influx. The effect of DIDS was observed in two experiments and the effect of K_o is representative of nine experiments

(pH 7.4 at 25°C) and then the suspensions were centrifuged, the supernatants were aspirated, and the cells were resuspended at the same hematocrit in identical media but with 1 mM KCl and 149 mM NaCl. The % nondiscs, determined as for previous experiments, increased from 11% at 90 mM K_o to 79% at 1 mM K_o, confirming that decreased K_o, hyperpolarization, or net K^+ efflux facilitates echinocytosis in cells treated with A23187. Control samples of cells that were centrifuged and resuspended in the same media at 90 mM K_o showed no significant change in the percentage of nondiscs. Echinocytes which had formed at 1 mM K_o did not revert to discs after resuspension at 90 mM K_o and incubation for 10 min, and were not observed for a longer period of time.

Ca Influx

Facilitation of Ca-induced echinocytosis in A23187-treated cells at low K_o, in comparison with null point K_o (Fig. 8), is associated with increased net uptake of Ca^{2+} (Fig. 9). In seven similar experiments, Ca uptake at 5 min was 4.0 ± 0.6 μmol/g Hb/5 min at 1 mM K_o and 2.8 ± 0.7 μmol/g Hb/5 min at 90 mM K_o, a significant increase ($P < 0.01$, 2-tailed student's t-test).. The difference of 1.2 μmol/g Hb corresponds to a maximal change in Ca_c of 600 μM, more than sufficient to account for the left-shift of the threshold for Ca-activated echinocytosis (Fig. 8). The actual increase in free Ca^{2+} is probably less due to intracellular buffers. In addition to increased Ca^{2+} uptake, loss of Mg^{2+} mediated by A23187 at low K_o might enable intracellular Ca^{2+} to compete more effectively at whatever site is responsible for echinocytosis.

The A23187-mediated Ca^{2+} influx (Fig. 9) is about 100-fold larger than the passive verapamil-sensitive influxes recently described by Varecka and Carafoli (1982) in cells in which the outward Ca^{2+} pump was inhibited by vanadate. With A23187, at both 1 and 90 mM K_o, Ca^{2+} influx was identical in the presence and absence of 100 μM verapamil (*not shown*).

The effect of K_o on A23187-mediated Ca^{2+} influx (Fig. 9, empty circles and squares) was first described by Sarkadi et al. (1976). Chlorobutanol, an inhibitor of Ca-activated K^+ conductance, effectively eliminated the extra Ca^{2+} uptake seen at low

Table. Evidence for electroneutral proton movements mediated by A23187 in DIDS-treated human red blood cells[a]

Ionophore	Inhibitor	K_o(mM)	Ca_o(μM)	ΔpH_o
A. Valinomycin		1	5	+0.03, +0.04
B. A23187		1	5	+0.13, +0.14
C. A23187		100	5	+0.11, +0.10
D. A23187	200 μM quinine	1	5	+0.10, +0.12
E. A23187	10 μg/ml oligomycin	1	5	+0.15, +0.10
F. A23187		1	500	−0.07, −0.15
G. A23187		1	1000	−0.13, −0.19

[a] Cells were washed three times in unbuffered 150 mM NaCl and treated at 25% HCT with 10 μM freshly prepared DIDS for 10 min at 23°C. Shown above are the changes in pH_o noted 1 min after addition of 1 μM valinomycin or 1.6 μM A23187 at the K_o and Ca_o indicated. The media contained 1 mM KCl and 149 mM NaCl, or in Row C 100 mM KCl and 50 mM NaCl. Ca_o was 5 μM, as measured by atomic absorption, except in the bottom 2 rows in which 0.5 or 1.0 mM $CaCl_2$ was added.

K_o (Sarkadi et al., 1976). Further insight into this phenomenon is provided by experiments in which the cells were treated with 10 μM DIDS, after which A23187-mediated Ca^{2+} uptake was independent of K_o and of E_m (Fig. 9, filled circles and squares). Such inhibition by DIDS of the extra component of Ca^{2+} influx in hyperpolarized cells (Fig. 9) suggests a linkage between A23187-mediated $Ca^{2+}/2H^+$ exchange and passive anion permeability. At low K_o, Ca^{2+}-induced K^+ conductance results in hyperpolarization which drives Cl^-, OH^- and HCO_3^- out of the cell in accordance with the redistribution required by the more negative E_m. After such net conductive loss of Cl^-, the inward Cl^- concentration gradient should be increased and the anion exchanger should then mediate electroneutral exchange of external Cl^- for intracellular OH^- or HCO_3^-. Since H^+ must be in equilibrium with OH^- and HCO_3^- inside and outside the cell, efflux of OH^- or HCO_3^- would leave excess intracellular H^+ to drive Ca^{2+} entry via A23187-mediated $Ca^{2+}/2H^+$ exchange. Since Cl^- conductance and exchange are both inhibited by DIDS, so also is that portion of A23187-mediated Ca^{2+} uptake which is driven by proton disequilibrium. The net result at low K_o is an apparent voltage dependence of Ca uptake which actually results from the parallel operation of electroneutral $Ca^{2+}/2H^+$ exchange, electroneutral Cl^-/HCO_3^- (OH^-) exchange, and Cl^- conductance.

Proton Movements

The validity of the explanation offered for the inhibition by DIDS of A23187-mediated Ca^{2+} uptake at low K_o (Fig. 9) depends on human red cells having a low proton conductance and on the Ca ionophore mediating primarily electroneutral exchange. Evidence supporting these concepts in human red cells is provided in the Table. In unbuffered media, addition of valinomycin results in a negligible external pH change, ΔpH_o, indicating low proton conductance (Table, row A). Subsequent addition of the proton ionophore FCCP permits larger external pH changes (Fig. 1), thus revealing the proton disequilibrium which exists upon hyperpolarization with valinomycin. With A23187 and no added Ca^{2+} or Mg^{2+}, but with the contaminating Ca^{2+} measured to be 5 μM by atomic absorption, the efflux of Mg^{2+} drives proton entry (Table, row B). Since 5 μM free Ca_o is sufficient to hyperpolarize the cells maximally, as may be inferred from Fig. 8, the effect of E_m on proton influx may be tested by raising K_o to 100 mM (Table, row C) or by inhibiting Ca-induced K^+ conductance with 200 μM quinine or with 10 μg/ml oligomycin (Table, rows D and E). By all three methods, inhibition of the hyperpolarization had no significant effect on ΔpH_o (Table, rows C–E). In contrast, raising external Ca^{2+} to 0.5 mM (Table, row F) or to 1.0 mM (Table, row G) changed the direction of proton movements from influx to efflux. Thus, the proton movements appear to be insensitive to changes in E_m but depend instead on the Mg^{2+} and Ca^{2+} gradients.

In summary the combination of low proton conductance and electroneutral $Ca^{2+}/2H^+$ exchange together result in an extra component of A23187-mediated Ca^{2+} uptake at low K_o in comparison with null point K_o (Fig. 9), an effect which perturbs the kinetics of Ca^{2+} uptake (Fig. 9) and which influences critically the threshold for Ca^{2+}-induced echinocytosis (Fig. 8).

Discussion

The experiments described in this paper were designed to evaluate whether or not changes in E_m of human red blood cells might affect cell shape. In isotonic DIDS-treated suspensions cell morphology

was unaltered by depolarization of +13 mV or by hyperpolarization of −82 mV, as induced by valinomycin at varied K_o (Fig. 3). Without DIDS, cell shape was similarly unaffected when E_m was varied with valinomycin (Fig. 4) or with gramicidin D (*not shown*). The effects noted with amphotericin B at low ionic strength (Fig. 6, bars *D* and *E*) were much less extensive than described by Glaser (1982), and were confounded by the effect of Na-deoxycholate in the pharmaceutical preparation of the drug (Fig. 5). Moreover, the absence of echinocytosis upon hyperpolarization with valinomycin at low ionic strength (Fig. 6, bars *H* and *I*) supports the conclusion that echinocytosis is independent of E_m. With A23187 at high Ca_o, Ca-induced echinocytosis proceeds independently of E_m (Fig. 7, top and middle rows), and a Ca_o was defined which gives maximal hyperpolarization at low K_o without a corresponding change in cell shape (Fig. 7, bottom row, and Fig. 8). While a more precise determination of the Ca_o thresholds for the Gardos effect and for echinocytosis could be made by means of a Ca^{2+}-sensitive electrode, the difference in the estimated thresholds (Fig. 8) also indicates that crenation is unrelated to E_m. The data do not exclude the possibility that stomatocytosis is associated with more extensive depolarization, but there is no compelling evidence that such is the case. The results (Figs. 1–3) do clearly demonstrate that the discocyte shape is compatible with membrane potentials of the order of −80 mV for at least 15 min.

Ca^{2+} entry promoted by A23187 stimulates the Ca-ATPase resulting in ATP depletion (Reed, 1976, 1979; Taylor, Baker & Hochstein, 1977). While early studies of metabolic depletion demonstrated that echinocytes form as ATP falls, and that the shape change is reversed upon ATP repletion with purine additives (Nakao et al., 1961), subsequent reports indicated that cell shape is not correlated with ATP concentration (Feo & Leblond, 1974), and that the discocyte form is compatible with the virtual absence of ATP, at least for short time periods (Feo & Mohandas, 1977). Whereas Ca^{2+} induces 100% echinocytosis within 15 min with A23187 (Fig. 6*C*), previous studies of incubations without glucose or ionophore indicate that ATP depletion, alteration of membrane lipids, and the resultant formation of echinocytes occur over a time course of hours (Shohet & Haley, 1973; Weed & Chailley, 1973), and without a Ca^{2+} requirement (Ferrell & Huestis, 1982).

The new observation that low K_o causes a left-shift of the Ca-activation curve for echinocytosis in cells treated with A23187 (Fig. 8) is consistent with the increased Ca uptake occurring at low K_o as compared to null-point K_o (Fig. 9, and Sarkadi et al., 1976). This effect was puzzling because A23187 was regarded as an electroneutral exchanger in rat red cells (Reed, 1976) and in other systems (Reed, 1979), and thus should not be affected by E_m. However, a small Ca^{2+} conductance was reported in A23187-doped planar lipid bilayers (Case, Vanderkooi & Scarpa, 1974), prompting us to examine the voltage dependence of A23187-mediated proton fluxes in human red cells. The data in the Table are consistent with A23187-mediated proton fluxes being insensitive to E_m but dependent on Ca^{2+} and Mg^{2+} gradients in human red cells. The inhibition of Ca^{2+} uptake at low K_o by DIDS (Fig. 9), despite the increased hyperpolarization upon inhibition of Cl^- conductance, indicates a coupling to anion transport. Thus Cl^-, HCO_3^-, and OH^- efflux in response to Ca-induced hyperpolarization would leave H^+ to drive an extra component of electroneutral A23187-mediated, DIDS-sensitive Ca^{2+} uptake. Further experiments would be needed in order to evaluate whether or not the hyperpolarization associated with the Gardos effect facilitates Ca uptake and echinocytosis in cells unexposed to A23187.

In summary, our observations agree qualitatively with those reported by Glaser (1979, 1982). However, additional experiments indicate that under the conditions studied, changes in E_m resulting in an altered strength of the electrical field across the membrane do not appear to counteract the forces which stabilize the shape of human red cells, forces which include lipid-lipid, protein-protein, and lipid-protein interactions.

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