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Inhibition of K^+ efflux and dehydration of sickle cells by [(dihydroindenyl)oxy]alkanoic acid: An inhibitor of the K^+Cl^- cotransport system

(ion transport/K^+ transport/cell volume/cell density/erythrocytes)

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ABSTRACT **[(Dihydroindenyl)oxy]alkanoic acid (DIOA) was recently introduced as a potent inhibitor of the K^+Cl^- cotransport system without side effects on other cation transport systems [Garay, R. P., Nazaret, C., Hannaert, P. A. & Cragoe, E. J., Jr. (1988) *Mol. Pharmacol.* 33, 696–701]. In sickle cells, an abnormal activation of this K^+Cl^- cotransport system was proposed to be involved in cell K^+ loss and dehydration. We found that DIOA inhibited the abnormal sickle cell K^+ loss and specifically reduced sickle cell density upon stimulation of the net outward K^+Cl^- cotransport—i.e., low pH, hypoosmolarity, and activation by *N*-ethylmaleimide. DIOA opens another therapeutic approach to sickle cell disease by inhibiting cell dehydration, which favors HbS polymerization and reduces erythrocyte deformability.**

In sickle cell anemia, the amount of HbS polymers and the delay time of HbS polymerization when deoxygenation occurs in the microvasculature are strongly dependent on the HbS concentration within erythrocytes (reviewed in refs. 1 and 2). It has also been shown that a proportion of sickle cells is abnormally dehydrated and exhibit a high Hb concentration (up to 50 g/dl). These dense sickle cells contain HbS polymers and are poorly deformable at relatively high Po_2. They are involved in the vasoocclusive crisis during which they are sequestered in the microvasculature (3). Consequently, the abnormal regulation of the water content of sickle cells plays an important role in the pathophysiology of sickle cell disease (4) and the inhibition of sickle cell dehydration should be of clinical benefit (5).

Sickle cell dehydration is related to a reduction of the concentration of monovalent cations within the erythrocytes, mainly a K^+ loss that is incompletely compensated by an increase in Na^+ (6, 7).

In 1971, Kregenow (ref. 8; see also ref. 9) reported that duck erythrocytes extrude K^+Cl^- (and water) in response to hypotonic medium. This phenomenon, subsequently found in erythrocytes from other species (including humans), apparently reflected the regulatory activity of a transport system able to catalyze outward and inward cotransport movements of K^+ and Cl^- and possesses the following properties: (*i*) the system was silent under physiological conditions and (*ii*) when the erythrocytes were swollen it used the energy of the electrochemical K^+ gradient to catalyze a net efflux of both K^+ and Cl^- thus helping the cell to extrude the excess intracellular water (9–12). In addition to hypotonicity, Cl^--dependent K^+ fluxes (ascribed to a K^+Cl^- cotransport system) could also be unmasked by the sulfhydryl group reagent *N*-ethylmaleimide (NEM) (11, 12). It is important to note that the activity of the human erythrocyte K^+Cl^- cotransport system decreases with cell aging (13, 14).

Different groups have reported that the K^+Cl^- cotransport system is abnormally activated in HbS and HbC cells and could be responsible for net K^+Cl^- extrusion and sickle cell dehydration (15–17).

Recently, Garay *et al.* (18) reported that [(dihydroindenyl)-oxy]alkanoic acid (DIOA) was able to potently inhibit K^+Cl^- cotransport fluxes without side effects on the bumetanide-sensitive Na^+,K^+Cl^- cotransport system. We therefore investigated the effect of DIOA on sickle cell K^+ loss and cell density.

METHODS

Blood was collected in heparinized Vacutainer tubes from informed adult patients homozygous for HbS and controls and was immediately processed. Plasma and buffy coat were removed by centrifugation at 1200 × *g* for 10 min and the cells were washed four times with a 150 mM NaCl solution at 4°C and suspended in this solution to obtain a hematocrit of 40%. The K^+ efflux was studied according to published methods (12, 15).

Briefly, the cells were diluted to a hematocrit of 2–3% and incubated with an incubation medium containing either 10 mM Tris Mops in the pH range 6.75–8 or Tris Mes at pH 6.25–6.50 at 37°C, 140 mM NaCl, 10 mM glucose, 1 mM $MgCl_2$, 0.1 mM ouabain, and 0.01 mM bumetanide. When the osmolarity was varied the medium contained 100 mM NaCl, 1 mM $MgCl_2$, 10 M glucose, 0.1 mM ouabain, 0.01 mM bumetamide, 10 mM Tris Mops (pH 7.4), and the osmolarity was varied from 220 to 400 mosM by adding choline chloride. The inhibitor DIOA was dissolved in dimethyl sulfoxide (DMSO) (10 mg per 0.5 ml) and was added at a final concentration of 100 μM unless otherwise stated. DMSO was added to a control sample. Ten milliliters of the incubation medium was added to 0.5 ml of the initial cell suspension and the mixture was distributed in a series of triplicate tubes, incubated at 37°C in a rotary shaking water bath. At 5-min or 30-min incubation times, the tubes were cooled in ice and centrifuged at 1200 × *g* for 5 min. The supernatant was evaluated for Hb and K^+ measured by atomic absorption using standards for K^+ (Varian AA 1275). The chloride-dependent K^+ efflux was the difference of the K^+ effluxes obtained in the presence of either sodium chloride or nitrate in the incubation medium. The NEM-stimulation of the K^+Cl^- cotransport was ob-

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Abbreviations: DIOA, [(dihydroindenyl)oxy]alkanoic acid; NEM, *N*-ethylmaleimide.

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tained by preincubating the cell suspension with 1 mM NEM. for 10 min at 0°C. Sickle cells were fractionated by stractan density gradient (19). The density distribution curve was performed by the phthalate ester method of Danon and Marikovsky (20).

RESULTS

In normal cells, the K^+ efflux was 2.17 ± 0.5 mmol per cell per hr in basal conditions (pH 7.4, isotonicity) and was not affected significantly by DIOA. In contrast, sickle cells exhibited an increased basal K^+ efflux 5.79 ± 1.33 mmol per cell per hr, which was reduced to 3.07 ± 1.38 mmol per cell per hr in the presence of DIOA (100 μM). The DIOA-sensitive K^+ efflux was highly increased in conditions that stimulate the K^+Cl^- cotransport system (Table 1). The IC_{50} of DIOA inhibition was similar to the values required for normal erythrocytes (1–3 × 10^{-5} M) and complete inhibition was reached with 8–10 × 10^{-5} M DIOA. All subsequent experiments were done with 10^{-4} M DIOA.

The pH-dependent K^+ efflux is a bell-shaped curve (Fig. 1) with a maximum between pH 6.7 and pH 7.0. In agreement with previous publications (12, 16), this pH-dependent K^+ efflux is highly increased in sickle cells in comparison to normal cells. In both cases, DIOA suppressed the pH stimulation of the K^+ efflux. At pH 6.25, an apparent K^+ efflux from sickle cells in the presence of DIOA is related to a small hemolysis as determined by the evaluation of the hemoglobin concentration of the supernatant.

Table 1 shows that DIOA inhibited the abnormal sickle cell K^+ loss induced by conditions (other than low pH) stimulating net outward K^+Cl^- cotransport—i.e., hypoosmolarity and activation by NEM. In addition, DIOA-sensitive K^+ efflux from sickle cells was chloride dependent.

Fig. 2 shows pH 7.0-stimulated K^+ efflux in light and dense sickle cells. In agreement with previous studies (16, 17), K^+ efflux at pH 7.0 was much higher in light than in dense sickle cells. In addition, Fig. 2 shows that DIOA strongly inhibited pH 7.0-stimulated K^+ efflux in light sickle cells.

Fig. 3 shows that a 2-hr incubation of sickle cells at pH 7.0 induced an increase in cell density and that DIOA prevented the density shift of all sickle cells.

DISCUSSION

The present report shows that the abnormally high K^+ efflux of sickle cells can be normalized *in vitro* by DIOA. This effect, due to the inhibition of net outward K^+Cl^- cotransport, prevented the density increase of HbS cells at pH 7.0.

It is known that the K^+Cl^- cotransport system is activated in reticulocytes (13, 14); therefore, reticulocytes could account for the increased activity of the K^+ efflux in sickle cells (21–23).

However, it has been shown that the high activity of the K^+Cl^- cotransport system in sickle cells cannot be attributed only to reticulocytes and the young cell age because this system is not correlated with the reticulocyte count (24) and is highly activated in erythrocytes of patients homozygous for HbC who have a moderate reduction of the erythrocyte life span and also in asymptomatic subjects heterozygous for

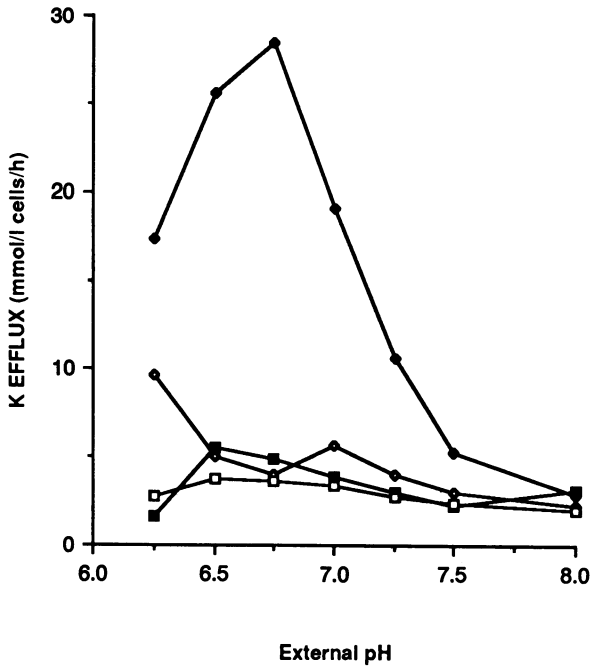

FIG. 1. Effect of DIOA on the pH-dependent K^+ efflux at 300 mosM. ◆, Sickle cells; ◇ sickle cells + DIOA; ■, normal cells; □, normal cells + DIOA.

HbC, who have normal reticulocyte count and erythrocyte survival but an active K^+Cl^- cotransport and an increased cell density (16, 17). Therefore, an additional mechanism to young erythrocyte age has to be invoked to explain the high activity of the K^+Cl^- cotransport in HbC cells and in sickle cells. HbS and HbC may have a direct or an indirect stimulating effect on the K^+Cl^- cotransport or on the maintenance of this cotransport during the aging process of erythrocytes.

The most interesting feature of this activated K^+Cl^- cotransport in sickle cells is its high sensitivity to pH changes. A slight reduction of the external pH as it occurs locally in many physiological or pathological conditions should increase the K^+ efflux. Low pH induces a fast and significant K^+Cl^- efflux, which is associated with a subsequent increase in density of most cells. The K^+Cl^- cotransport of sickle cells is permanently activated at a low rate at pH 7.40 but at a much faster rate at pH 7.2 or 7.0 (12). This could explain why a proportion of sickle cells are dehydrated *in vivo*. In addition, reversible sequestration of erythrocytes in microvasculature by lowering the local pH should further increase the K^+Cl^- cotransport, the erythrocyte dehydration, and recruit progressively more cells to contain polymers. The K^+Cl^- cotransport inhibitor DIOA prevents the formation of dense cells induced by incubating sickle cells at pH 7.0.

In this context, DIOA is of interest to define the K^+ efflux due to the K^+Cl^- cotransport and a DIOA-resistant K^+ efflux. In the presence of ouabain and bumetanide, which block the Na^+,K^+ pump and the Na^+,K^+Cl^- cotransport, respectively, DIOA does not inhibit completely the K^+

Table 1. Effect of DIOA on volume and NEM-stimulated K^+ efflux (at pH 7.4) and on Cl^--dependent K^+ efflux (stimulated at pH 7.0) of sickle cells

	Hypoosmolarity (220 mosM) (pH 7.4)	NEM (pH 7.4)	$NaNO_3$ (*a*) (pH 7.0)	NaCl (*b*) (pH 7.0)	Cl^--dependent K^+ efflux (*b* − *a*) (pH 7.0)
Control	21.00 ± 0.75	19.80 ± 0.55	4.08 ± 0.26	18.17 ± 0.15	14.09 ± 0.41
DIOA (100 μM)	4.95 ± 0.20	4.90 ± 0.35	3.14 ± 0.31	4.79 ± 0.44	1.65 ± 0.75

Cl^--dependent K^+ efflux was estimated as the difference between 140 mM NaCl (*b*) and 140 mM $NaNO_3$ (*a*).

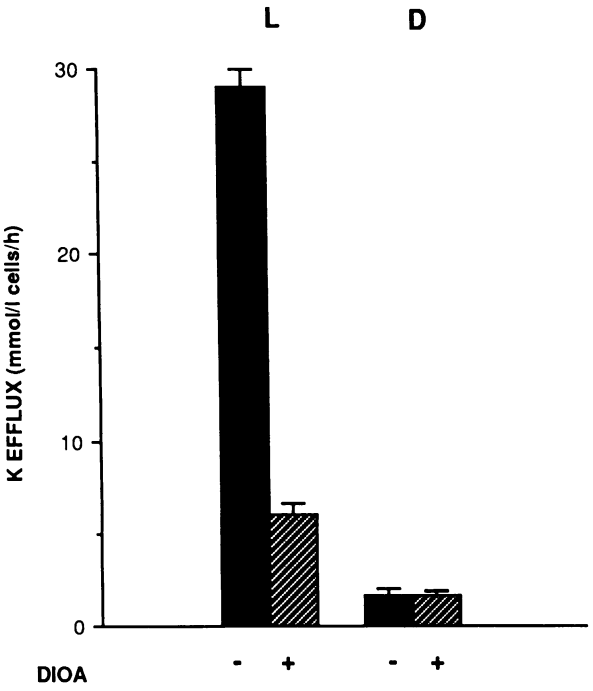

FIG. 2. Effect of DIOA on the K^+ efflux of sickle cells fractionated by stractan density gradient. L, light cells: $1.077 < d < 1.088$. D, dense cells: $d > 1.096$.

efflux. DIOA normalizes the K^+ efflux in sickle cells activated by either hypoosmolarity, low pH, or NEM. However, a low K^+ efflux remains when the three inhibitors are used in combination. The DIOA-resistant K^+ efflux is the same in normal and in sickle cells. The DIOA-resistant K^+ efflux may represent basal diffusion of K^+ or another still uncharacterized K^+ channel that is not chloride dependent.

The DIOA inhibition of sickle cell dehydration induced at pH 7.0 suggested by the density distribution curve (Fig. 3) is related to the inhibition of the K^+Cl^- cotransport system by DIOA and not to a significant increase of Na^+ in cells incubated with DIOA (O.O., unpublished data).

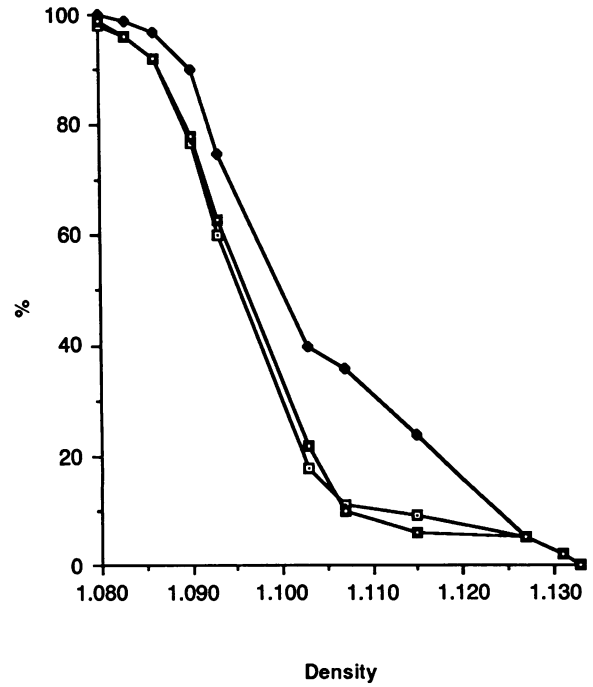

FIG. 3. Density distribution curves of sickle cells incubated at pH 7.0 for 120 min with DIOA (⊡), without DIOA (♦), and control without DIOA and not incubated (□). The concentration of 2,3-diphosphoglycerate was not affected during the incubation.

In addition, DIOA is of special interest in designing a therapy for sickle cell disease because it prevents one of the major defects of sickle cells, which is their tendency to dehydrate. The DIOA effect is increased when sickle cells are exposed to a lower external pH, occurring frequently in physiological conditions in kidney, during muscular exercise, and in many pathological conditions that favor general or local acidosis: hypoxemia, fever, sequestration in the microvasculature, etc. It is possible that with time the DIOA inhibitory activity of K^+Cl^- cotransport may not only restore the cell volume of dehydrated cells but also swell most of the sickle cells, decreasing the HbS concentration. The sickle cell swelling should in turn reduce the proportion of cells containing polymers in the arterial side of the microvasculature, prevent the formation of abnormally dense sickle cells, and consequently improve the blood viscosity, the cell deformability, and also decrease the critical Po_2 at which a given cell will form hemoglobin polymers during the time required to pass through the capillaries.

In conclusion, DIOA is potentially important, because by inhibiting the activated K^+Cl^- cotransport system it opens a therapeutic approach to sickle cell disease and other pathological conditions that maintain or activate this K^+Cl^- cotransport system. However, other compounds of the same series, toxicity, and bioavailability have not yet been studied. In addition, this type of compound with more potent inhibitory activity than DIOA could be useful to purify the K^+Cl^- cotransport system.

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