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Demonstration of a [K^+,Cl^-]-Cotransport System in Human Red Cells by Its Sensitivity to [(Dihydroindenyl)oxy]alkanoic Acids: Regulation of Cell Swelling and Distinction from the Bumetanide-Sensitive [Na^+,K^+,Cl^-]-Cotransport System

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SUMMARY

A screening of several families of compounds on NEM-stimulated K^+ efflux in human red cells allowed us to select a [(dihydroindenyl)oxy] alkanoic acid (DIOA) as the first potent inhibitor of this K^+ flux (IC_{50} of 10^{-5} M) without side effects on the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system. Incubation of human red cells in hypotonic media (179 mosm) increased cell volume (by 18–20%) and provoked the appearance of a DIOA-sensitive K^+ efflux of 4.48 ± 0.83 mmol·(liter of cells × hr)$^{-1}$ (mean ± SD of nine experiments). This DIOA-sensitive K^+ efflux exhibited a Michaelian-like dependence on the Cl^- concentration of the incubation media (freely equilibrated with intracellular Cl^-) with an apparent dissociation constant of 39.6 ± 14.7 mM and a maximal rate of 4.7 ± 0.9 mmol·(liter of cells × hr)$^{-1}$ (mean ± SD of five experiments). The chloride effect was mediated by intracellular and not by extracellular Cl^-, as expected for an outward [K^+,Cl^-]-cotransport. The above properties of DIOA-sensitive K^+ efflux clearly confirm that human red cells have a [K^+,Cl^-]-cotransport system that regulates cell swelling. The regulatory response to hypotonic media was also strongly depressed by cytochalasin B at a concentration of 1 mM, suggesting that the activating signal is probably transduced by the cytoskeleton.

In 1971 Kregenow reported that duck red cells extrude KCl (and water) in response to hypotonic media (1; see also Ref. 2). 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 possessing the following properties: (i) the system was silent under physiological conditions and (ii) when the red cells were swollen it used the energy of the electrochemical K^+ gradient in order to catalyze a net efflux of both K^+ and Cl^-, thus helping the cell to extrude the excess intracellular water (2–8). In addition to hypotonicity, Cl^--dependent K^+ fluxes (ascribed to a [K^+,Cl^-]-cotransport system) could also be unmasked by the sulfhydryl group reagent NEM (6–8).

On the other hand, the use of loop diuretics, particularly bumetanide, allowed the clear characterization of a membrane [Na^+,K^+,Cl^-]-cotransport system in erythrocytes and several other cells (for recent reviews on the [Na^+,K^+,Cl^-]-cotransport system see Refs. 8–11). It is interesting to note that the mechanism of action of these loop diuretics, which are carboxylic acids, involves a competition with chloride for a common anionic receptor site on the [Na^+,K^+,Cl^-]-cotransport system (12).

In contrast to the [Na^+,K^+,Cl^-]-cotransport system, NEM-stimulated K^+ fluxes were only inhibited by high concentrations of bumetanide (7). This and other indirect evidence led most authors to assume that the Cl^--dependent K^+ fluxes, unmasked by hypotonic media or NEM, were catalyzed by a [K^+,Cl^-]-cotransport system different from the [Na^+,K^+,Cl^-]-cotransport system (8).

It appeared to us that one way to clearly demonstrate the existence of a membrane [K^+,Cl^-]-cotransport system, different from the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system, was to develop a potent (and specific) inhibitory compound. Therefore, during the past few years we have tested several families of compounds on NEM-stimulated K^+ fluxes (and on the [Na^+,K^+,Cl^-]-cotransport system) in human red cells. Our guiding strategy was based in the screening of compounds having the following potential activities: (i) carboxylic or sulfonic acids, which may act at the Cl^--site of the [K^+,Cl^-]-cotransporter; (ii) cinchona alkaloids, amiloride, and other organic cations, which may act at the K^+-site of the [K^+,Cl^-]-cotransporter; and (iii) diuretics, antihypertensives, or other pharmacological agents used in transport-linked pathologies.

The above screening showed that four [(dihydroindenyl)oxy]

ABBREVIATIONS: NEM, *N*-ethylmaleimide; MOPS 4-morpholinopropanesulfonic acid; DIOA, [(dihydroindenyl)oxy]alkanoic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DMSO, dimethyl sulfoxide.

alkanoic acids (see structures in Table 1) were able to potently inhibit NEM-stimulated K^+ efflux without side effects on the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system. The use of one of these inhibitors (DIOA) clearly showed that human red cell membranes have a [K^+,Cl^-]-cotransport system that regulates erythrocyte swelling.

Methods

NEM-stimulated K^+ efflux. NEM-stimulated K^+ efflux in human red cells was measured by a method essentially similar to the one previously published (7). Briefly, erythrocytes were washed three times with 150 mM cold NaCl. After the last wash, the cells were suspended at a hematocrit of 8–10% in two tubes containing K^+-Ringer's medium with and without 0.5 mM NEM. The K^+-Ringer's medium contained (mM): 135 KCl, 15 NaCl, 1 $MgCl_2$, 2.5 potassium phosphate buffer (pH 7.4 at 37°), and 10 glucose. The osmolality was adjusted to 295 ± 5 mosm. Both cell suspensions were incubated for 15 min at 37°. After this incubation period, the cells were washed five times with 110 mM cold $MgCl_2$. After the last wash, the cells were suspended in Mg-sucrose medium at a hematocrit of 5–7%. The Mg-sucrose medium contained (mM): 75 $MgCl_2$, 85 sucrose, 10 MOPS-Tris (pH 7.4 at 37°), and 10 glucose (the osmolality was adjusted to 295 ± 5 mosm). A portion of each cell suspension was set aside to measure hematocrit, intracellular Na^+ and K^+ by flame photometry, and hemoglobin absorbance by spectrophotometry.

A volume of 0.5 ml of the cell suspensions in Mg-sucrose medium was added to two tubes containing 2 ml of Mg-sucrose medium with 10 μM bumetanide and 100 μM ouabain (final hematocrit was between 1 and 1.4%). The tubes were incubated for 30 min at 37°. At the end of the incubation period the tubes were chilled at 4° for 1 min and then centrifuged at 1750 × *g* for 4 min at 4°. The supernatants were transferred into tubes for K^+ analysis in an Eppendorf flame photometer. K^+ standards (checked with commercial standards; Merck, Darmstadt, FRG) were prepared in water and compared with those prepared in the different efflux media. In control experiments no evidence of red cell lysis during the incubation in the efflux media could be detected. NEM-stimulated K^+ efflux was calculated from the difference in K^+ efflux between tubes with and without NEM. In some experiments NEM-stimulated K^+ efflux in isotonic Mg^{2+}-sucrose was compared with that measured in Na^+,Rb^+-Ringer's medium of the following composition (mM): 140 NaCl, 5 RbCl, 1 $MgCl_2$, 10 MOPS-Tris (pH 7.4 at 37°C), 0.1 ouabain, 0.01 bumetanide, and 10 glucose.

The effect of drugs, hypotonicity, and Cl^- substitution. The compounds used in the study have been described previously (13–15). The structures of the [(dihydroindenyl)oxy]alkanoic acids are shown in Table 1. The compounds that bear a common name are labeled accordingly. The remaining compounds are given the code number used in previous publications (13, 16). The absolute configuration and optical rotation is noted for each chiral compound. The free acid (or base) form was neutralized with Tris base (or with MOPS).

To study the effects of several compounds on NEM-stimulated K^+ efflux in human erythrocytes, the compounds were added from freshly prepared concentrated stock solutions in water, ethanol, or DMSO, provided that the final concentrations of these solvents had no effect per se on ion transport (final DMSO concentration in the flux media was always lower than 0.86%). In particular, [(dihydroindenyl)oxy] alkanoic acids were tested from stock solutions containing 50–500 mM of compounds in DMSO. All drugs were tested in concentration-response curves.

In experiments with hyposmotic media, ouabain- and bumetanide-resistant K^+ efflux was studied in media with different concentrations of NaCl using a protocol similar to that described above. Briefly, fresh erythrocytes were washed (three times) and resuspended with 150 mM cold NaCl at a hematocrit of 20–25%. A volume of 0.5 ml of the cell suspension was added to duplicates of tubes containing 2 ml of Na^+-Ringer's media of the following composition (mM): (140 − *x*) NaCl, 5 RbCl, 1 $MgCl_2$, 10 MOPS-Tris (pH 7.4 at 37°), 0.1 ouabain, 0.01 bumetanide, and 10 glucose, where *x* varied from 0 to 60 mM. The osmolality of the solutions was measured by using a Knauer Semi-micro-osmometer (Oberursel, FRG). The tubes were incubated for 0, 30, and 60 min at 37° and K^+ efflux was measured as before.

To study the effect of Cl^- substitution, ouabain- and bumetanide-resistant K^+ efflux was studied in fresh erythrocytes washed three times with 150 mM $NaNO_3$ and resuspended in Na^+-Ringer's media in which different amounts of Cl^- were substituted mole by mole with NO_3^-. K^+ efflux was measured as before (for further details see legends to figures).

Results

Inhibition of NEM-stimulated K^+ efflux by compounds. We found that NEM-stimulated K^+ efflux was much more resistant to drugs than fluxes catalyzed by the [Na^+,K^+,Cl^-]-cotransport system (and by other erythrocyte ion transport systems). Fig. 1 shows that only certain carboxylic acids, particularly four [(dihydroindenyl)oxy]alkanoic acids were active on NEM-stimulated K^+ efflux. The structures and IC_{50} of these [(dihydroindenyl)oxy]alkanoic acids are shown in Table 1. Fig. 1 shows that, of the remaining carboxylic acids tested, only the loop diuretics furosemide and ethacrynic acid and the anti-brain edema agent 5c(+) (see Ref. 16 for chemical structures) were capable of inhibiting NEM-stimulated K^+ fluxes and this at high concentrations (IC_{50} of about 10^{-3} M or higher). Even higher concentrations were required for inhibiting NEM-stimulated K^+ efflux with organic cations or neutral diuretics (for instance amiloride or hydrochlorothiazide, at concentrations of 5×10^{-3} M, only inhibited 20–25% of NEM-stimulated K^+ efflux; Fig. 1).

Among the compounds that were inactive on NEM-stimulated K^+ fluxes it is interesting to mention the following: (i) muzolimine (Specia Laboratories, Paris, France), a loop diuretic acting on the serosal side of Henle's loop (17), where a [K^+,Cl^-]cotransport system has been suggested (18); (ii) ciclotanine (I.H.B. Research Laboratory, Le Plessis, France), a furopyridine diuretic previously found to stimulate ouabain- and bumetanide-resistant K^+ fluxes in human red cells (19); and (iii) quinidine, an inhibitor of Ca^{2+}-dependent K^+ channels (tested up to a concentration of 10^{-4} M because it increases nonspecific membrane cation leak). Finally, it should be noted that high concentrations of DIDS (a potent inhibitor of the Cl^-/HCO_3^- anion exchanger) partially inhibited NEM-stimulated K^+ efflux (about 30% inhibition between 10^{-4} and 10^{-3} M).

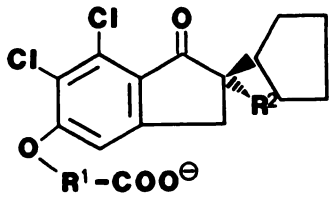
Investigation of [K^+,Cl^-]-cotransport fluxes by using [(dihydroindenyl)oxy]alkanoic acids. The above results clearly showed that [(dihydroindenyl)oxy]alkanoic acids, particularly (*R*-(+)-8.4 A were potent inhibitors of NEM-stimulated K^+ efflux in human red cells (see Table 1). In addition, we have previously found that they have few or no side effect on [Na^+,K^+,Cl^-]-cotransport fluxes (Ref. 16) (Table 1).

For the sake of simplicity (*R*-(+)-8.4 A is given the code name DIOA. This compound inhibited NEM-stimulated K^+ efflux with a mean IC_{50} of $1.0 \pm 0.1 \times 10^{-5}$ M (mean ± SE of six experiments performed in Mg-sucrose medium; see Table 1 and Fig. 1). It is important to note that, in additional experiments measuring NEM-stimulated K^+ efflux in Na^+,Rb^+-Ringer's medium, DIOA exhibited similar IC_{50} values ($0.91 \pm 0.23 \times 10^{-5}$ M, mean ± SE of five experiments). Moreover, a similar

TABLE 1

Inhibition of erythrocyte Cl-transport systems by dihydroindenyloxyalkanoic acids

Values are given as means of three to six experiments.



Compound	R¹	R²	IC_{50} [K⁺,Cl⁻]-Cotransport	IC_{50} [Na⁺,K⁺,Cl⁻]-Cotransport[a]	IC_{50} Cl^-/HCO_3^- Anion exchanger[a]
			M		
(*R*)-(+)-8.4 A	—CH_2—	—$(CH_2)_3$—CH_3	10^{-5}	No effect	10^{-5}
(*R*)-(+)-8.10 A	—$(CH_2)_3$—	—CH_3	3×10^{-5}	No effect	8×10^{-5}
(±)-8.2[b]	—CH_2—	—CH_3	10^{-4}	10^{-4}	4×10^{-5}
(*S*)-(−)-8.13 B	—$(CH_2)_3$—	—$(CH_2)_3$—CH_3	~10^{-4}	No effect	10^{-5}

[a] From Ref. 16.
[b] Racemic mixture.

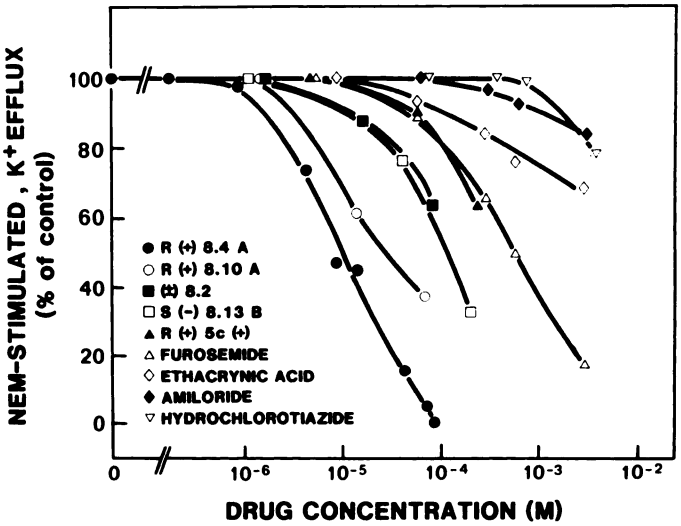


Fig. 1. Inhibition of NEM-stimulated K^+ efflux by compounds in human red blood cells. Each dose-response curve represents mean values of three to six experiments (the ranges were almost similar in size to the experimental points). It can be seen that only certain carboxylic acids, particularly four [(dihydroindenyl)oxy]alkanoic acids inhibited NEM-stimulated K^+ efflux (see structures in Table 1). Higher concentrations of the remaining compounds (the carboxylic acids furosemide and ethacrynic acid and the anti-brain edema agent 5c(+), the organic cation amiloride, or the diuretic hydrochlorothiazide; see Refs. 13, 16, 21, and 22 for chemical structures) were required to inhibit NEM-stimulated K^+ fluxes. Most of the tested compounds were inactive in this assay, i.e., muzolimine, cicletanine, and quinidine (see text).

IC_{50} of about 10^{-5} M was obtained in experiments in which DIOA was tested on a hyposmotically induced K^+ efflux.

Almost all tested drugs, at high concentrations, were able to increase the nonspecific membrane K^+ leak (a prehemolytic effect). Regarding DIOA, Table 2 shows that this toxic effect appeared at concentrations of about 172 μM and that a DIOA concentration of 344 μM stimulated a K^+ leak that was similar in magnitude to the NEM-stimulated K^+ efflux. Therefore we used a DIOA concentration of 86 μM in order to completely inhibit [K^+,Cl^-]-cotransport fluxes without increasing the nonspecific membrane K^+ leak.

Unmasking of DIOA-sensitive K^+ fluxes by red cell swelling. The stimulation of outward [K^+,Cl^-]-cotransport fluxes by a hyposmotic stress was examined first. Fig. 2 shows that, similar to erythrocytes from duck and other species (2, 4, 5), K^+ efflux was increased in human red cells incubated in hyposmotic media and that DIOA completely abolished this phenomenon. A careful inspection of Fig. 2 shows that in isotonic media the system was almost silent (DIOA-sensitive K^+ efflux was between 0.1 and 0.6 mmol·(liter of cells × hr)$^{-1}$). Conversely, when the external osmolality was reduced to 179 mosm, DIOA-sensitive K^+ efflux was stimulated up to 4.48 ± 0.83 mmol·(liter cells × hr)$^{-1}$ (mean ± SD of nine healthy subjects). This procedure induced an 18–20% increase in erythrocyte volume (measured as in Refs. 7 and 16), which resulted therefore in a 4–5% loss of cell KCl per hour.

Besides hypotonicity, another experimental condition inducing erythrocyte swelling is acidification (20). We have found that erythrocyte acidification induced a DIOA-sensitive K^+ efflux of about 2 mmol·(liter of cells × hr)$^{-1}$ with a half-maximal stimulation at a pH of 7.0 (Na^+ efflux remained unchanged). However, it is important to note that this phenomenon was also inhibited by 10 μM DIDS (which blocks chloride movements through the anion carrier).

The effect of Cl^- substitution on DIOA-sensitive K^+ fluxes. The next series of experiments was designed to investigate the Cl^--dependence of DIOA-sensitive K^+ efflux. Fig. 3 shows DIOA-sensitive K^+ efflux as a function of the Cl^- concentration in the incubation media ($[Cl^-]_o$). It is important to

TABLE 2

Nonspecific K^+ leak induced by DIOA in human red cells

Values of total K^+ leak are given as mean ± standard deviation. The number of experiments is indicated in parentheses.

DIOA	Total K^+ leak	DIOA-stimulated K^+ leak	
μM	*mmol·(liter of cells × hr)*$^{-1}$	*mmol·(liter of cells × hr)*$^{-1}$	% of [K^+,Cl^-]co-transport fluxes[a]
None	2.15 ± 0.51 (11)		
86	1.77 ± 0.41 (11)	NS[b]	0.0
172	3.67 ± 0.90 (4)	1.52[c]	25.3
344	10.1 ± 5.2 (4)	7.95[c]	132.5

[a] An estimation of the relative significance of DIOA-stimulated K^+ leak was done by comparing it with a mean value of [K^+,Cl^-]-cotransport fluxes of 6.0 mmol·(liter of cells × hr)$^{-1}$ (taken from Ref. 7 and from this study).
[b] NS, not significant, Student's *t* test.
[c] $p < 0.01$, Student's *t* test.

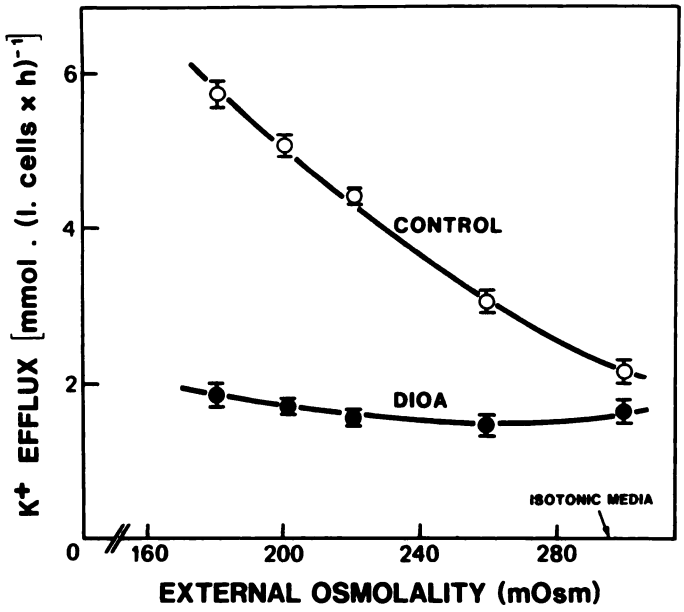


Fig. 2. Stimulation of erythrocyte K^+ efflux by hyposmotic stress and full inhibition by a [(dihydroindenyl)oxy]alkanoic acid (DIOA; (*R*)-(+)-8.4A in Table 1). Values are given with their range as a measure of variability. DIOA-sensitive K^+ efflux was almost nonexistent in isotonic media. Conversely, when the external osmolality was reduced to 179 mosm, DIOA-sensitive K^+ efflux was strongly stimulated up to about 4 mmol·(liter of cells × hr)$^{-1}$. Similar results were obtained in eight other experiments.

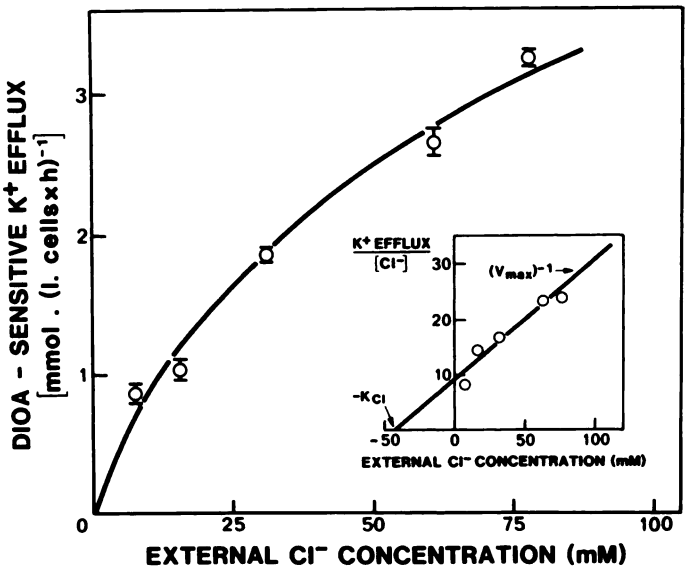


Fig. 3. DIOA-sensitive K^+ efflux as a function of the Cl^- concentration in the incubation media ($[Cl^-]_o$). NaCl was replaced by $NaNO_3$ and the total ($NaNO_3$ plus NaCl) concentration was maintained constant at approximately 89 mM. The cells were preequilibrated with NO_3^- and Cl^- (freely diffusing through the anion carrier and by passive permeability). DIOA was used at a concentration of 86 μM. Values are given with their range as a measure of variability. Similar results were obtained in four other experiments. *Inset*, Hanes plot of the data. An apparent dissociation constant for $[Cl^-]_o$ (K_{Cl}) of about 42 mM was obtained from the intercept with the *x* axis and a maximal rate (V_{max}) of 4.6 mmol·(liter of cells × hr)$^{-1}$ from the reciprocal of the slope.

outline that in replacing NaCl with $NaNO_3$, the total ($NaNO_3$ plus NaCl) concentration was maintained constant at approximately 89 mM and that the cells were preequilibrated with NO_3^- and Cl^- (moving across the red cell membrane through the anion carrier and by passive permeability). Fig. 3 (*inset*) shows a Hanes plot of the data. The straight line that resulted strongly suggested that DIOA-sensitive K^+ efflux was a Michaelian-like function of $[Cl^-]_o$. The apparent dissociation constant for $[Cl^-]$ (K_{Cl}) was obtained from the intercept with the *x* axis and the maximal rate (V_{max}) from the reciprocal of the slope (Fig. 3, *inset*). In erythrocytes from five different healthy blood donors K_{Cl} was 39.6 ± 14.7 mM and V_{max} was 4.7 ± 0.9 mmol·(liter of cells × hr)$^{-1}$ (mean ± SD).

We explored *cis*- and *trans*-effects of chloride on DIOA-sensitive K^+ efflux. Our main result was that, as expected for an outward $[K^+,Cl^-]$-cotransport flux, DIOA-sensitive K^+ efflux was *cis*-activated by intracellular Cl^- ($[Cl^-]_i$) and not *trans*-activated by $[Cl^-]_o$ (i.e., DIOA-sensitive K^+ efflux was completely lacking in erythrocytes in which $[Cl^-]_i$ was replaced by NO_3^-, subsequently washed with a DIDS-containing NO_3^- solution, and incubated in Cl^--media containing DIDS).

We investigated whether the carboxylic acid DIOA inhibited outward $[K^+,Cl^-]$-cotransport fluxes at the Cl^- site, by developing dose-response curves for this compound at different Cl^- concentrations. Unfortunately, as shown in Fig. 4, we were unable to determine IC_{50} values in low-Cl^- media because increasing DIAO concentrations in such low-Cl^- media initially stimulated and then inhibited DIOA-sensitive K^+ efflux.

Inhibition of $[K^+,Cl^-]$-cotransport fluxes by cytochalasin B. Fig. 5 shows that cytochalasin B strongly depressed the hyposmotically induced DIOA-sensitive K^+ efflux. It is important to emphasize the fact that we used high cytochalasin B concentrations (1 mM) because erythrocytes are less sensitive to this drug than are epithelia.

Discussion

The study of membrane ion transport was greatly facilitated by the discovery of potent and quite specific inhibitory drugs. A first generation of transport inhibitors was readily discovered because these compounds were therapeutic agents, like the cardiac glycosides are for the Na^+,K^+-pump and loop diuretics (furosemide and bumetanide) are for the $[Na^+,K^+,Cl^-]$-cotransport system. Conversely, the development of inhibitors for

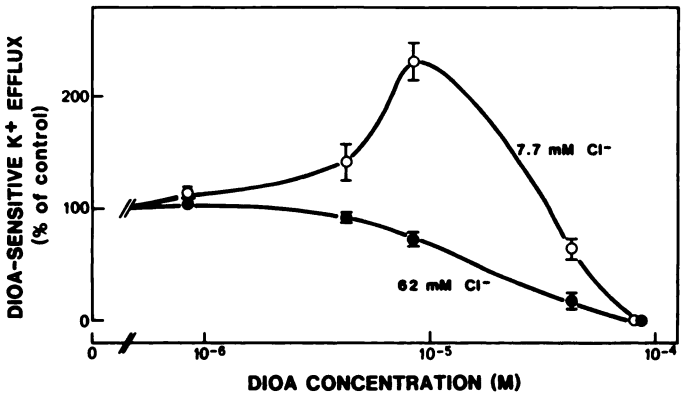


Fig. 4. Stimulation of $[K^+,Cl^-]$-cotransport fluxes by DIOA at low chloride concentrations. A 2- to 3-fold stimulation of DIOA-sensitive K^+ efflux was seen with 10^{-6} M DIOA at a chloride concentration of 7.7 mM. Similar results were obtained in four other experiments.

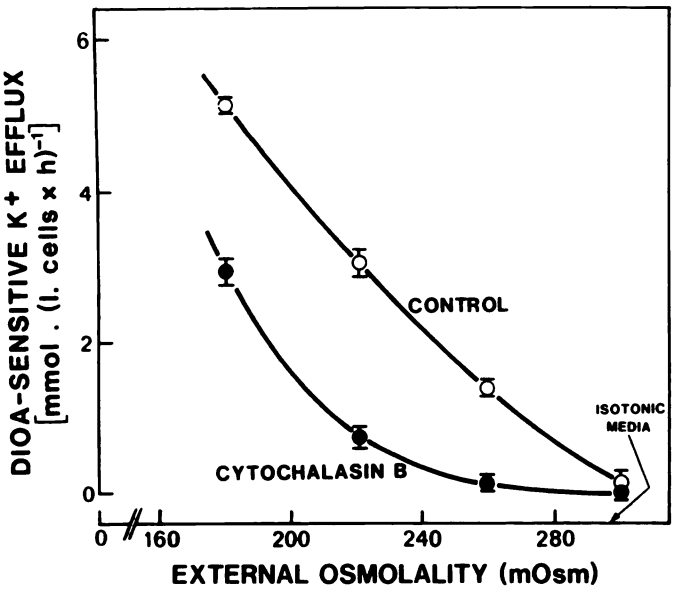


Fig. 5. Inhibition by cytochalasin B of the regulatory response of the DIAO-sensitive [K^+,Cl^-]-cotransport system to hyposmotic stress. The erythrocytes were preincubated for 15 min at 37° with 1 mM cytochalasin B and then further incubated during the flux period with the same cythocalasin B concentration. Similar results were obtained in two other experiments.

other transport systems such as Na^+,Ca^{2+} and Na^+,H^+ exchangers required the screening of large numbers of molecules (see for instance Refs. 21 and 22).

Indirect evidence has previously suggested that the Cl^--dependent K^+ fluxes, unmasked by hypotonic media and by NEM, are catalyzed by a [K^+,Cl^-]-cotransport system different from the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system (see for instance Refs. 7 and 8). Therefore, we were interested in discovering an inhibitor of NEM-stimulated K^+ efflux without side effects on the [Na^+,K^+,Cl^-]-cotransport system. To expedite the screening of molecules, we used the precise and rapid assay of NEM-stimulated K^+ efflux in human red cells. Unfortunately, NEM-stimulated K^+ efflux was resistant to a number of treatment modalities. This obliged us to screen a large number of drugs before observing significant inhibition with the [(dihydroindenyl)oxy]alkanoic acids. These compounds are indane analogues of ethacrynic acid that have lost both diuretic activity and inhibitory activity on the [Na^+,K^+,Cl^-]-cotransport system (13, 16). This latter property was crucial because it permitted the discrimination between [K^+,Cl^-]- and [Na^+,K^+,Cl^-]-cotransport fluxes (Table 1).

We supposed that, similarly to loop diuretics for the [Na^+,K^+,Cl^-]-cotransport system, the mechanism of action of [(dihydroindenyl)oxy]alkanoic acids may involve competition with Cl^- for a common site on the [K^+,Cl^-]-cotransport system. Unfortunately, with media low in Cl^- we were unable to determine IC_{50} values because increasing DIAO concentrations initially stimulated and then inhibited DIOA-sensitive K^+ efflux (Fig. 4). This suggests that low DIOA concentrations may displace Cl^- from a regulatory site, as previously reported for furosemide on the anion carrier (23). Therefore, similar to the anion carrier, the [K^+,Cl^-]-cotransport system could have both regulatory and catalytic Cl^- sites. Whether Cl^- and/or other anionic effectors can modulate the activity of the [K^+,Cl^-]-cotransport system (and cell volume) at a regulatory site is a matter for further investigation.

Lauf (6) has previously found in sheep red cells that external Rb^+ increases the apparent affinity of NEM-stimulated K^+ efflux for furosemide. However, this was not the case for [(dihydroindenyl)oxy]alkanoic acids, suggesting that the sites at which these compounds and furosemide act on the [K^+,Cl^-]-cotransport system may be overlapping, but not identical.

Of course [(dihydroindenyl)oxy]alkanoic acids, which are carboxylic acids, can be potential inhibitors of other Cl^- transport systems. Indeed, we have previously observed that, similar to several other carboxylic acids, [(dihydroindenyl)oxy]alkanoic acids are able to inhibit the DIDS-sensitive Cl^-/HCO_3^- erythrocyte anion exchanger (Ref. 16 and Table 1). However, this side effect was not important for our purposes because the anion carrier does not catalyze KCO_3^- movements (24; see also Ref. 25) and because the study was conducted in the absence of added HCO_3^-. Indeed, furosemide was used for almost 15 years to characterize the [Na^+,K^+,Cl^-]-cotransport system (see for instance a recent use in Ref. 26), in spite of the fact that it also effectively inhibits the anion carrier (23). On the other hand, Table 1 shows among other things that decreasing the number of C-atoms in the alkyl R^2 residue, i.e., the (*R*)-(+)-8.10 A analogue, increases by about 3-fold the inhibitory activity on NEM-stimulated K^+ efflux as compared with that on Cl^-/HCO_3^- exchange. This can be used as a guiding criterion for the chemical synthesis of an inhibitory analogue without side effects on the anion carrier.

High concentrations of (*R*)-(+)-8.4 A induced an increase in the nonspecific membrane K^+ leak (a prehemolytic effect). In addition, the range of (*R*)-(+)-8.4 A concentrations for complete inhibition of NEM-stimulated K^+ efflux without important side effects on the K^+ leak was very narrow (≈86–172 μM; Table 2). Again, a parallel could be established with the narrow range of furosemide used for inhibiting the [Na^+,K^+,Cl^-]-cotransport system (an inhibitory concentration of 1 mM was commonly used, whereas the compound induced an increase in K^+ leak at concentrations of 2 mM).

Based on the above considerations, we selected (*R*)-(+)-8.4 A (Table 1) as a useful tool for reinvestigating the possible existence and physiological role of an erythrocyte [K^+,Cl^-]-cotransport system, which is different from the well established bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system. For the sake of simplicity, we gave this particular [(dihydroindenyl)oxy]alkanoic acid the code name DIOA. Of course, future screening studies may allow the development of a better inhibitor than DIOA (without side effects on the anion carrier and with a better range between inhibitory and toxic doses). Indeed, such kind of improvements allowed the introduction of bumetanide for replacing furosemide in studies of the [Na^+,K^+,Cl^-]-cotransport system.

DIOA completely abolished the KCl loss of human erythrocytes in response to hypotonic stress. A DIOA-sensitive, 4–5% cell KCl loss per hour was found after an 18–20% increase in erythrocyte volume, as expected for a regulator of erythrocyte swelling. Interestingly, DIOA-sensitive K^+ fluxes were 1 to 2 orders of magnitude higher than the K^+ fluxes catalyzed by the [Na^+,K^+,Cl^-]-cotransport system (19), a transport system without apparent physiological role in human red blood cells.

DIOA also inhibited a K^+ efflux induced by erythrocyte acidification (and its consecutive cell swelling). However, the participation of the [K^+,Cl^-]-cotransport system in this phenomenon requires further investigation because (i) the anion

carrier is involved in the cell swelling that follows erythrocyte acidification (20); (ii) K^+ efflux induced by erythrocyte acidification was also inhibited by DIDS; (iii) DIOA is also able to inhibit the anion carrier (Ref. 16 and Table 1); and (iv) in low-K^+ sheep red cells, DIDS stimulates K^+ efflux (27).

The only Cl^- fluxes that can be accurately measured in human red blood cells are those catalyzed by the anion carrier. To our knowledge, no one has been able to determine Cl^- fluxes catalyzed by the [Na^+,K^+,Cl^-]-cotransport system, even when the anion carrier was blocked with DIDS (measurement of Cl^- fluxes catalyzed by the [Na^+,K^+,Cl^-]-cotransport system was performed on red cells from other species; see for instance Ref. 28). Therefore, we tried to adapt an alternate procedure, previously used by several authors for the characterization of co- or countertransport mechanisms, i.e., to study *cis-* and *trans-*effects of co- or counterions. This clearly showed that DIAO-sensitive K^+ efflux was *cis*-stimulated by Cl^- as expected for an outward [K^+,Cl^-]-cotransport movement.

The results obtained with DIOA show that the erythrocyte swelling induced by a variety of pathophysiological circumstances unmasks a compensatory KCl extrusion through a [K^+,Cl^-]-cotransport system. However, this regulatory behavior raises the intriguing question as to how a membrane transport system may sense that the cell is swollen. We suspected a possible participation of the cytoskeleton and we tested the effect of cytochalasin B. This compound induced a partial suppression of the regulatory properties of the [K^+,Cl^-]-cotransport system, suggesting that erythrocyte swelling may unmask the activity of the [K^+,Cl^-]-cotransport system through an interaction with the cytoskeleton (Fig. 5). However, further experiments are required in order to confirm this interaction because, besides the action on the cytoskeleton, cytochalasin B interacts with the glucose carrier and other membrane proteins.

In conclusion, [(dihydroindenyl)oxy]alkanoic acids (particularly DIOA) are potent inhibitors of NEM-stimulated K^+ efflux in human red cells without side effects on the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system. The study of DIOA-sensitive K^+ movements clearly confirmed that human red cell membranes possess a [K^+,Cl^-]-cotransport system that regulates cell swelling. The signal for the swelling-dependent activation of this transport system seems to be transduced by the cytoskeleton. We anticipate that the use of DIOA for characterizing the [K^+,Cl^-]-cotransport system may help to understand physiological and physiopathological aspects of cell volume regulation in the near future.

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