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Chloride-activated passive potassium transport in human erythrocytes

(erythrocyte membranes/cation transport/cation permeability)

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ABSTRACT **Passive K^+ transport in human erythrocytes (defined as ouabain-insensitive transport) was inhibited 70% by replacement of Cl^- by several permeant monovalent anions. The V_{max} of Cl^--dependent K^+ influx was 1.14 mmol·liter^{-1}·hr^{-1}; its apparent K_m for K^+ was 4.7 mM. There was a much smaller component of Na^+ influx dependent on Cl^- (V_{max}, 0.23 mmol·liter^{-1}·hr^{-1}). Furosemide and other inhibitors of Cl^- transport inhibited passive K^+ transport to the same extent as replacement of Cl^-, but 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, a specific inhibitor of anion exchange in erythrocytes, was ineffective. The Cl^--dependent K^+ transport, which may be K^+/Cl^- cotransport, could reflect a mechanism for regulating cell volume.**

Human erythrocytes have at least four transport systems for K^+ to cross the membrane: the Na^+/K^+ pump (1), the Ca^{2+}-activated transport system (or Gardos effect) (2), the Na^+/K^+ cotransport system (3–5), and a nonspecific electrodiffusional leak (6). In sheep erythrocytes there is an additional passive K^+ transport system that is sensitive to changes in cell volume: K^+ fluxes increase in swollen cells and decrease in shrunken cells. This K^+ transport system requires the presence of chloride for its operation (7). In the present paper we report the Cl^- dependence of passive K^+ transport in human erythrocytes (passive transport taken to mean a process not inhibited by ouabain). We show the kinetics of the Cl^--activated influx with regard to extracellular concentrations of K^+ and Cl^-, $[K]_o$ and $[Cl]_o$. Passive Na^+ influx was also dependent on Cl^-, but the maximal velocity was one-fifth that for K^+. Finally, we present results on pharmacological agents and Cl^--activated K^+ influx. Of particular interest is furosemide which, in erythrocytes, inhibits both Cl^- transport (8) and Na^+/K^+ cotransport (5).

MATERIALS AND METHODS

Cells. Blood was drawn by venipuncture from healthy adult donors. The erythrocytes were washed three times in an isotonic saline solution by centrifugation and resuspension. The saline solution contained 150 mM NaCl, 5 mM glucose, 10 mM Hepes at pH 7.5 (adjusted at 20°C with NaOH). In all experiments the cells used had been obtained the same day.

Chloride Replacement. In order to study the effects of varying $[Cl]_o$, we equilibrated cells in media with various permeant anions at 150 mM substituted for Cl^- in the isotonic saline. The cells were first washed in a medium with the same composition as the isotonic saline (except for the substituted anion) and incubated at 37°C for 30 min. They were then washed again, incubated another 30 min, and finally washed three more times.

Fluxes. Unidirectional influxes were measured as described (9) with ^{24}Na, ^{22}Na, or ^{86}Rb (the latter as a tracer for K^+). The incubations with the tracer were for 30 min. The Cl^- added with the tracer was ignored (final concentration ≈ 1 μM). The method used for unidirectional K^+ efflux has been described (10); ^{42}K was used as a tracer. Na^+ efflux was measured by the same method. All fluxes are expressed as mmol per liter of packed cells per hr. Errors are given as SEM. Passive K^+ transport was taken as that measured in cells incubated with ouabain (0.05 mM). When the effect of furosemide was to be determined, cells were preincubated with the drug for 5 min before addition of the tracer.

Reagents. Choline chloride, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), Hepes, and sodium and potassium methylsulfates were obtained from British Drug Houses (Poole, Dorset, England). Choline chloride was recrystallized from a hot ethanol solution. Ouabain was obtained from Sigma. Furosemide and 3-*N*-pyrrolidino-4-phenoxy-5-sulfamoylbenzoic acid (piretanide) were gifts from Hoechst Pharmaceuticals (Hounslow, Middlesex, England). 6,7-Dichloro-2-methyl-2-phenyl-1-oxoindanyloxyl acid (MK-196) was a gift from Merck Sharp & Dohme (Hoddesden, Hertfordshire, England). All other compounds were analytical reagent grade.

RESULTS

Passive K^+ Influx with Replacement of Cl^-. Fig. 1 shows passive K^+ influxes in cells equilibrated in media with Cl^- replaced by a series of monovalent, permeant anions. With all substitute anions tested, K^+ influx was inhibited. This effect was only partial with bromide, but significant (at least 70% inhibition) with all the other substitutes. Furosemide (1 mM) inhibited K^+ influx to about the same extent as Cl^- replacement, but caused no further inhibition when added to Cl^--free medium (see below).

K^+ Influx and $[Cl]_o$. Fig. 2 shows the dependence of passive K^+ influx on $[Cl]_o$. In two separate experiments Cl^- was replaced by either methylsulfate or nitrate with essentially the same result: activation of K^+ influx by increasing $[Cl]_o$. These results, together with those in Fig. 1, make it clear that the inhibition observed is a consequence of the removal of Cl^- and is not caused by pharmacological effects of the substituted anions.

The kinetics of the activation of K^+ influx by Cl^- are not simple; the curve relating influx to $[Cl]_o$ is sigmoid, suggesting either the action of Cl^- at multiple sites or the involvement of secondary effects. In any case, the interpretation of these results

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Abbreviations: $[X]_o$, concentration of solute X in a medium for suspending cells; piretanide, 3-*N*-pyrrolidino-4-phenoxy-5-sulfamoylbenzoic acid; MK-196, 6,7-dichloro-2-methyl-2-phenyl-1-oxoindanyloxyl acetic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

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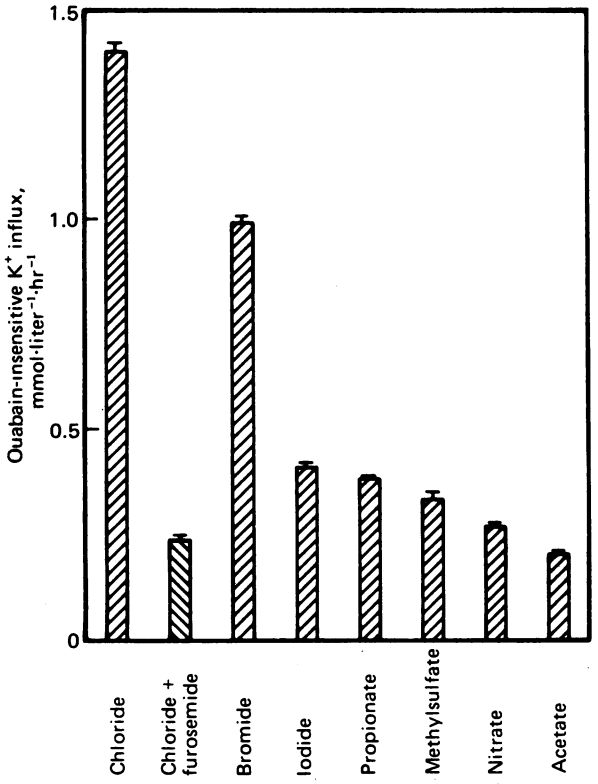

FIG. 1. Unidirectional passive K^+ influxes in human erythrocytes equilibrated in a medium with Cl^- or in Cl^--free media with various substitute anions (at 150 mM). The cells were equilibrated in these media and fluxes were measured in media with 15 mM K^+ and 0.05 mM ouabain (n = 4). The concentration of furosemide was 1 mM.

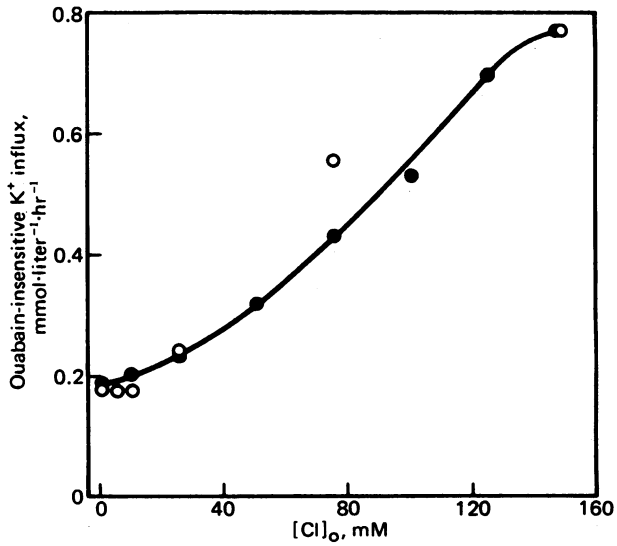

FIG. 2. Passive K^+ influx in human erythrocytes with varying $[Cl]_o$. The cells were equilibrated in media with the various Cl^- concentrations (0–150 mM), the anion content being made up by either methylsulfate (○) or nitrate (●). All solutions contained, during the flux measurements, 15 mM K^+ and 0.05 mM ouabain. The SEMs, if shown, would all be smaller than the symbols (n = 3, methylsulfate; n = 4, nitrate). The active (ouabain-inhibitable) K^+ influx did not vary with $[Cl]_o$: 1.60 ± 0.03 mmol·liter of cells^{-1}·hr^{-1} (n = 6) in the experiment with methylsulfate. Likewise, the flux measured with both ouabain and furosemide (1 mM) was constant with varying $[Cl]_o$: 0.16 ± 0.01 mmol·liter^{-1}·hr^{-1}, methylsulfate experiment (n = 6). The curve was fitted by eye.

is complicated by the necessity of varying both intracellular and external concentrations of the anions, where transmembrane effects may be involved.

In the experiment in Fig. 2, active K^+ influx (ouabain-inhibitable) was simultaneously determined and was unaffected by varying $[Cl]_o$, as was the influx measured with ouabain and furosemide together.

Passive K^+ efflux was also inhibited by replacement of Cl^-. In a typical experiment, K^+ efflux was 3.57 ± 0.35 mmol·liter^{-1}·hr^{-1} from cells in Cl^- medium, and 1.84 ± 0.19 in a medium with methylsulfate replacing Cl^- (n = 3), intracellular K^+ being 88 mmol per liter of packed cells. The effluxes were measured in K^+-free media containing 145 mM NaCl or Na methylsulfate.

K^+ Influx and $[K]_o$. Fig. 3 shows the dependence of passive K^+ influx on $[K]_o$ in cells in normal (high $[Cl]_o$) and Cl^--free (NO_3^--substituted) media. Because choline nitrate was not readily available, in the NO_3^- medium over the concentration range $[K]_o$ = 0–50 mM, $[Na]_o$ was varied reciprocally from 150 to 100 mM. To control for possible effects of Na^+ (see below), we determined fluxes in Cl^- media both at a fixed $[Na]_o$ of 100 mM (choline replacement of K^+) and a variable $[Na]_o$ of 100–150 mM, yielding identical results (Fig. 3). The data presented in Fig. 3 clearly demonstrate two components of passive K^+ transport: a saturable system dependent on Cl^- and a nonsaturable system independent of Cl^-, in NO_3^- (Cl^--free) media. The data for the influx in the presence of Cl^- are fit well by an equation that assumes these two components of K^+ influx. The equation is the sum of a linear component (Cl^- independent), given by the product of a proportionality constant and $[K]_o$, and a saturating (hyperbolic) Cl^--dependent component, given by a simple Michaelis function (see legend, Fig. 3). The V_{max} (maximum velocity) of the Cl^--dependent K^+ influx was 1.14 mmol·liter^{-1}·hr^{-1}. Its apparent Michaelis constant was 4.7 mM.

Na^+ Flux with Replacement of Cl^-. In an initial series of experiments, the effect of replacement of Cl^- on Na^+ influx was determined by simultaneously measuring Na^+ and K^+ influx from an isotonic medium with equimolar concentrations of Na^+ and K^+ (75 mM) and with either Cl^- or NO_3^- as the principal anion. Because there was a small but significant effect of Cl^- removal on Na^+ influx, the experiment was repeated at $[Na]_o$ of 15 mM ($[K]_o$ = 135 mM). At the lower concentration, the saturable influx would be a larger fraction of total influx. As shown in Table 1, there was clearly an inhibition of Na^+ entry by either Cl^- substitution or by furosemide, and the two types of inhibition were not additive. Experiments on Na^+ efflux confirmed a Cl^--dependent Na^+ transport: from cells in a Cl^- medium the flux was 0.52 ± 0.006 mmol·liter^{-1}·hr^{-1}, and from cells in a NO_3^- medium it was 0.26 ± 0.005 (n = 3). Cellular Na^+ concentration was 5.4 mmol per liter of cells. The effluxes were measured in media containing 135 mM KCl, 15 mM NaCl, and 0.05 mM ouabain, the same as the media in the experiments in Table 1.

The kinetics of the dependence of Na^+ influx on $[Cl]_o$ were determined in cells equilibrated in a series of media with $[Cl]_o$ and $[NO_3]_o$ varied reciprocally (Fig. 4). There was activation of Na^+ influx by increasing $[Cl]_o$, with an indication of saturation as $[Cl]_o$ approached 80 mM. Apparently about half of Na^+ influx is dependent on Cl^-, in contrast to up to 80% of passive K^+ influx.

Fig. 5 shows the kinetics of Na^+ influx as $[Na]_o$ was varied. In Cl^- media $[K]_o$ was both held constant and varied, just as $[Na]_o$ was in the experiment in Fig. 3. Na^+ influx was the same whether $[K]_o$ was constant at 100 mM or varied between 100 and 150 mM. The Na^+ influx in the NO_3^- (Cl^--free) media was

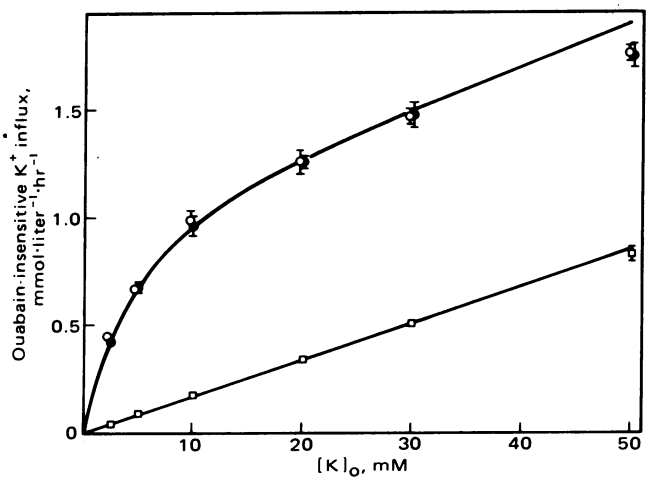

FIG. 3. Passive K^+ influx with varying $[K]_o$ in human erythrocytes equilibrated in media with either Cl^- (○, ●) or NO_3^- (□) as the principal anion (150 mM). In Cl^- media, as $[K]_o$ was varied from 0 to 50 mM, $[Na]_o$ was either kept constant at 100 mM (●) or varied from 150 to 100 mM (○). When $[Na]_o$ was constant, K^+ was replaced with choline. In the NO_3^- media (□), $[Na]_o$ was varied. Ouabain was in all media at 0.05 mM. In Cl^- media the dependence of K^+ influx on $[K]_o$ was the same whether $[Na]_o$ was constant or varied. The line through the upper points, for Cl^- media, was calculated from:

$$M = \frac{M_{max}}{1 + (K_m/[K]_o)} + \alpha[K]_o,$$

in which M is passive K^+ influx, M_{max} is maximal K^+ influx (1.14 mmol·liter^{-1}·hr^{-1}), K_m is the apparent Michaelis constant of the saturable component of influx (4.7 mM), and α is the proportionality constant (0.017) relating $[K]_o$ to the nonsaturating K^+ influx obtained in NO_3^- media (□). Error bars were omitted when they were smaller than the symbols (n = 3).

not quite linear with increasing $[Na]_o$, but showed slight saturation. Therefore, in calculating the curve drawn through the points for the Cl^- media, the equation included a term for the Na^+ influx in NO_3^- media measured at each value of $[Na]_o$ (see legend, Fig. 5). It was not possible to use a proportionality constant multiplied by $[Na]_o$, the procedure used for passive K^+ influx in Cl^--free media (Fig. 3). In Cl^- media, Na^+ influx had an additional saturable component that fit well to the Michaelis function with a maximum velocity of 0.23 mmol·liter^{-1}·hr^{-1} and an apparent Michaelis constant of 7.7 mM. The maximal velocity was about one-fifth that for K^+. The apparent K_m was similar to that for K^+, about 60% higher. It should be emphasized that the conditions for determining the kinetic constants were different for Na^+ influx and for K^+ influx. $[Na]_o$ was 100 mM for the K^+ fluxes; $[K]_o$ was 100 mM for the Na^+ fluxes. There may be inhibitory or activating effects (or both) of one ion on the transport of the other.

Table 1. Dependence of Na^+ influx on $[Cl]_o$ in human erythrocytes

Anion	Ouabain-insensitive Na^+ influx, mmol·liter^{-1}·hr^{-1}
Cl^-	0.468 ± 0.002
Cl^- (+ furosemide)	0.214 ± 0.010
NO_3^-	0.219 ± 0.002
NO_3^- (+ furosemide)	0.206 ± 0.006

Cells were equilibrated in a medium with either Cl^- or NO_3^- as the primary anion (150 mM). The fluxes were measured at $[Na]_o$ = 15 mM, $[K]_o$ = 135 mM, and with ouabain at 0.05 mM. Furosemide, when present, was at 1 mM (n = 4).

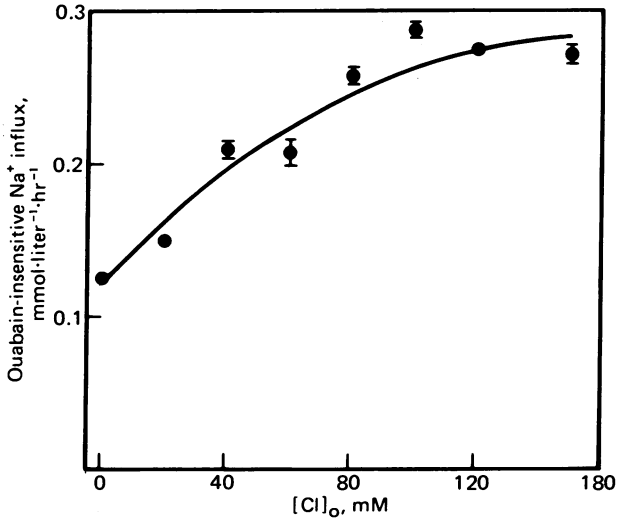

FIG. 4. Na^+ influx in human erythrocytes with varying $[Cl]_o$. The cells were equilibrated in media with various Cl^- concentrations (0–150 mM), the anion content being made up by nitrate. All solutions contained, during the flux measurements, 15 mM K^+ and 0.05 mM ouabain. Error bars were omitted when they were smaller than the symbols (n = 3). The curve was fitted by eye.

A fraction of Na^+ influx could be as the ionic species $NaCO_3^-$ exchanging for intracellular anion (11); K^+ does not form the corresponding ionic species. Because the exchanges of $NaCO_3^-$ for NO_3^- would be slower than for Cl^-, the present results on the dependence of Na^+ influx on Cl^- should be interpreted cautiously until the involvement of $NaCO_3^-$ is determined. In this context previous results with sheep erythrocytes failed to show any effect of replacing Cl^- on Na^+ influx (9).

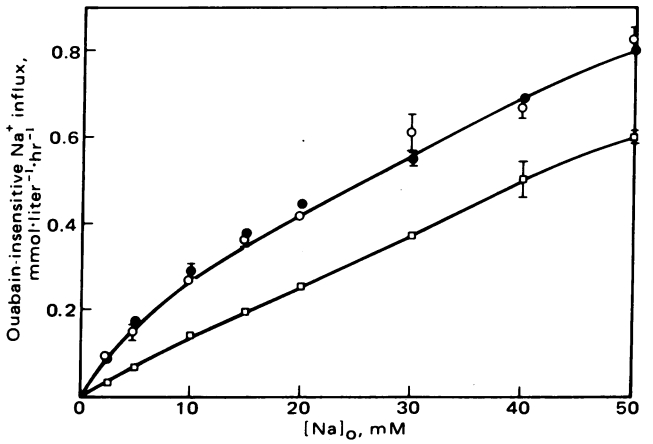

FIG. 5. Na^+ influx with varying $[Na]_o$ in human erythrocytes equilibrated in media with either Cl^- (○, ●) or NO_3^- (□) as the principal anion (150 mM). In the Cl^- media, as $[Na]_o$ was varied from 0 to 50, mM $[K]_o$ was either kept constant at 100 mM (●) or varied from 150 to 100 mM (○). When $[K]_o$ was constant, Na^+ was replaced with choline. In the NO_3^- media (□), $[K]_o$ was varied. Ouabain was in all media at 0.05 mM. In the Cl^- media the dependence of Na^+ influx on $[Na]_o$ was the same whether $[K]_o$ was constant or varied. The line through the upper points, for Cl^- media, was calculated from:

$$M = \frac{M_{max}}{1 + (K_m/[Na]_o)} + D,$$

in which M is Na^+ influx, M_{max} is maximal Na^+ influx (0.232 mmol·liter^{-1}·hr^{-1}), K_m is the apparent Michaelis constant (7.7 mM), and D is the Na^+ influx in NO_3^- medium measured at that $[Na]_o$ (□). Error bars were omitted when they were smaller than the symbols (n = 3).

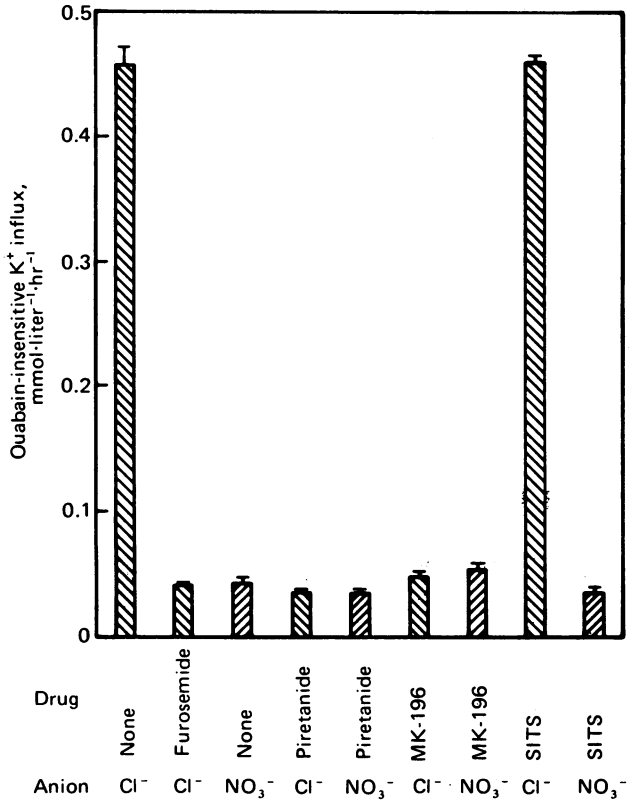

FIG. 6. Effects of pharmacological agents on passive K^+ influx in human erythrocytes equilibrated in media containing either Cl^- or NO_3^- (150 mM). Fluxes were measured in a medium with ouabain (0.05 mM), K^+ (2.5 mM), Na^+ (147.5 mM), and drugs as indicated: furosemide (1 mM), piretanide (1 mM), MK-196 (0.2 mM), and SITS (0.1 mM) (n = 3).

K^+ Influx and Inhibitors of Cl^- Transport. Fig. 6 shows the effects of various pharmacological agents that inhibit anion fluxes in erythrocytes (12). All of these agents inhibited the K^+ influx, except for SITS, a specific inhibitor of anion exchange mediated by an identified membrane protein in "band 3" (13). Therefore, the Cl^--activated K^+ transport is not associated with the protein in band 3 which is responsible for anion exchange.

DISCUSSION

The present results show that most of the passive (i.e., ouabain-insensitive) K^+ influx in human erythrocytes requires chloride. There was an earlier observation of an effect in human erythrocytes that is probably the same phenomenon. Funder and Wieth (14) observed a slight inhibition (14%) of passive K^+ influx with most of the Cl^- replaced by 120 mM thiocyanate ($[Cl]_o$ = 6.5 mM) and a marked inhibition with 120 mM iodide (57%), nitrate (54%), or bicarbonate (72%). (The Cl^- medium as well as the substituted media contained 22 mM HCO_3^-.) There was no inhibition with 120 mM bromide. They also carried out flux ratio analysis of passive K^+ transport in cells in media with the various anions. The measured flux ratios corresponded well to the predicted ratios for all anions except chloride and bromide. Based upon these observations, Funder and Wieth (14) speculated that there may be a transport mechanism operating only with Cl^- and Br^-, accounting for the lower fluxes and the agreement of measured and predicted flux ratios with the other anions. The authors did not characterize this transport system further.

There are some apparent discrepancies between our results and those of Funder and Wieth (14). First of all, they observed an increase (33%) in Na^+ influx when NO_3^- was substituted for Cl^-. Some preliminary results on the interaction between Na^+ and K^+ in the Cl^--activated transport system enable us to resolve the discrepancy. Our experiments in Table 1 were carried out with a high $[K]_o$ (135 mM), and those of Funder and Wieth at a low $[K]_o$ (3.7 mM). We have recently confirmed that at low $[K]_o$, NO_3^- increases Na^+ influx; that raising $[K]_o$ in Cl^- media increases Na influx; and that at high $[K]_o$ (>26 mM), NO_3^- decreases Na^+ influx. Finally, raising $[K]_o$ in NO_3^- media causes a slight inhibition of Na^+ influx (15). Therefore, the Cl^--activated Na^+ influx requires K^+, and the apparent Na^+/K^+ cotransport is associated with it.

The second discrepancy is the slight increase in K^+ efflux that Funder and Wieth (14) observed after replacing Cl^- with NO_3^- or I^-. We observed a decrease in K^+ efflux under similar conditions. We cannot really resolve this discrepancy, though the decrease in efflux we observed was less than the decrease in influx, and a net efflux would result.

Activation of passive K^+ influx by Cl^- and saturation kinetics for K^+ are consistent with cotransport of K^+ and Cl^-, but measurements have not been made of the flux of Cl^-. Cotransport of K^+ and Cl^- has been proposed for avian erythrocytes (16) and Ehrlich ascites tumor cells (17) and may be involved in regulation of the volume of cells. Activation of K^+ influx by Cl^- of course does not prove cotransport. The striking difference in kinetics of the activation of passive K^+ influx by K^+ (hyperbolic) and by Cl^- (sigmoid) suggests a complex system, whether it be cotransport or activation by Cl^- with no accompanying Cl^- flux.

The Cl^--activated K^+ influx in human erythrocytes is superficially similar to the system previously described as a K^+ transport dependent on Cl^- and also on cell volume in sheep erythrocytes (7). We found that in human erythrocytes, there is no effect of volume in K^+ influx (7). This confirmed the early finding of Davson (18). There was a more recent report of an increase in K^+ transport with decreased volume in human erythrocytes (19). Though the conditions of the measurements were different, there is no obvious way to account for the different results. The volume-dependent, Cl^--activated K^+ influx in sheep cells was specific for K^+ in that there was no effect on Na^+ influx of varying either cell volume or $[Cl]_o$. Thus, the specificity of the Cl^--dependent cation influx differs between sheep and human erythrocytes, there being Cl^--dependent Na^+ influx in human cells. The difference is consistent with the observation that there is no dependence of passive K^+ influx on Na^+ in sheep cells (20), and therefore no Na^+/K^+ cotransport.

Furosemide inhibits the Cl^--dependent influxes of K^+ and Na^+ in human erythrocytes (Fig. 1 and Table 1) and also inhibits Cl^- transport (8) and Na^+/K^+ cotransport (5). It is not clear if the Cl^--dependent K^+ influx is related to the Na^+/K^+ cotransport. On the one hand, both are inhibited by furosemide. On the other hand, Na^+ seems to have a lower maximal velocity (compare Figs. 3 and 5) for the system than does K^+, although transported with a reasonably high affinity. Furthermore, Wiley and Cooper (5) do show a substantial furosemide-sensitive K^+ influx in Na^+-free medium which may also be Cl^- sensitive.

Piretanide and MK-196, as well as furosemide, inhibited the Cl^--dependent K^+ system. These compounds inhibit anion exchange in erythrocytes (12), but unlike SITS, they inhibit other membrane transport systems as well (e.g., ref. 21). Their action on passive K^+ transport may reflect an inhibition at a Cl^--binding site of the K^+ transport system (see ref. 8).

We show here significant Cl^--dependent K^+ influx (V_{max}, 1.14 mmol·liter^{-1}·hr^{-1}) and Na^+ influx (V_{max}, 0.23 mmol·liter^{-1}·hr^{-1}). The relationship between these three species of ion (K^+, Na^+, and Cl^-) remains unsettled. Further experiments directed toward determining whether it is possible to show a K^+- or Na^+-dependent Cl^- transport through the furosemide-sensitive pathway should resolve this issue.

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