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Furosemide-Sensitive K^+ (Rb^+) Transport in Human Erythrocytes: Modes of Operation, Dependence on Extracellular and Intracellular Na^+, Kinetics, pH Dependency and the Effect of Cell Volume and N-Ethylmaleimide

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Summary. The effect of extracellular and intracellular Na^+ (Na_o^+, Na_i^+) on ouabain-resistant, furosemide-sensitive (FS) Rb^+ transport was studied in human erythrocytes under varying experimental conditions. The results obtained are consistent with the view that a (1 Na^+ + 1 K^+ + 2 Cl^-) cotransport system operates in two different modes: mode *i*) promoting bidirectional 1 : 1 (Na^+-K^+) cotransport, and mode *ii*) a Na_o^+-independent 1 : 1 K_o^+/K_i^+ exchange requiring Na_i^+ which, however, is not extruded. The activities of the two modes of operation vary strictly in parallel to each other among erythrocytes of different donors and in cell fractions of individual donors separated according to density. Rb^+ uptake through Rb_o^+/K_i^+ exchange contributes about 25% to total Rb^+ uptake in 145 mM NaCl media containing 5 mM RbCl at normal Na_i^+ (pH 7.4). Na^+-K^+ cotransport into the cells occurs largely additive to K^+/K^+ exchange. Inward Na^+-Rb^+ cotransport exhibits a substrate inhibition at high Rb_o^+. With increasing pH, the maximum rate of cotransport is accelerated at the expense of K^+/K^+ exchange (apparent pK close to pH 7.4). The apparent $K_m Rb_o^+$ of Na^+-K^+ cotransport is low (2 mM) and almost independent of pH, and high for K^+/K^+ exchange (10 to 15 mM), the affinity increasing with pH. The two modes are discussed in terms of a partial reaction scheme of (1 Na^+ + 1 K^+ + 2 Cl^-) cotransport with ordered binding and debinding, exhibiting a glide symmetry (first on outside = first off inside) as proposed by McManus for duck erythrocytes (McManus, T.J., 1987, *Fed. Proc., in press*). N-ethylmaleimide (NEM) chemically induces a Cl^--dependent K^+ transport pathway that is independent of both Na_o^+ and Na_i^+. This pathway differs in many properties from the basal, Na_o^+-independent K^+/K^+ exchange active in untreated human erythrocytes at normal cell volume. Cell swelling accelerates a Na_o^+-independent FS K^+ transport pathway which most probably is not identical to basal K^+/K^+ exchange. $K_o^+ < Na_o^+ < Li_o^+ < Mg_o^{2+}$ reduce furosemide-resistant Rb^+ inward leakage relative to $choline_o^+$.

Key Words red blood cells · Na^+-K^+ cotransport · K^+/K^+ exchange · Cl^--dependent K^+ transport · red cell age

Introduction

Ouabain-resistant inward and outward transport of both Na^+ and K^+ in human erythrocytes show saturating components. These are mediated by the Na^+/Na^+ (Na^+/Li^+) exchange system [13] and by the so-called Na^+-K^+ cotransport system [44] which is dependent on chloride [7, 21] and blocked by loop diuretics such as furosemide or bumetanide [3, 5, 8, 14, 17, 23, 43, 44].

The cotransport system of human erythrocytes has often been thought to mediate an electroneutral, bidirectional symport of (1 Na^+ + 1 K^+ + 2 Cl^-), as observed with duck erythrocytes [28] and Ehrlich cells [24]. However, Na^+ to K^+ stoichiometries varying between 1 : 1 and 1 : 5 have been reported [3, 5, 21, 23, 44]. Furthermore, furosemide-sensitive (FS) and chloride-dependent inward movements of K^+ have been described that occur in pure choline media in the absence of extracellular Na^+ (Na_o^+) [5, 8, 21, 30, 43, 44]. Thus, FS K^+ movements in human erythrocytes cannot simply be attributed to the action of a strictly coupled Na^+-K^+ cotransport system. In addition, the sulfhydryl reagent N-ethylmaleimide (NEM) is known to activate a Cl^--dependent K^+ transport pathway which is normally silent. This pathway is independent of Na_o^+ and partially inhibited by furosemide or bumetanide [11, 18, 32, 36–38, 43].

In duck erythrocytes, three different modes of cotransport sensitive to inhibition by loop diuretics seem to exist: the first mediates quaternary cotransport (1 Na^+ + 1 K^+ + 2 Cl^-), the second 1 : 1 K_o^+/K_i^+ exchange activated by Na_i^+, and the third binary cotransport (1 K^+ + 1 Cl^-) independent of both Na_o^+ and Na_i^+. The first two modes are activated by cell shrinkage, cAMP and catecholamines, and the third by cell swelling [26–29, 39,–41]. Na^+-K^+ cotransport is also accelerated in shrunken rat erythrocytes [12, 15], and enhancements of Na_o^+-independent (Cl^--dependent) K^+ movements have been observed in swollen low K^+ sheep [19, 34, 35] and rat erythrocytes [12]. The accelerations of chloride-dependent K^+ movements reported to occur in swollen human erythrocytes [1, 2, 12, 18, 30, 31] are possibly involved in a regulatory volume decrease.

Table 1. Effect of 100 mM extracellular Na^+ and furosemide (0.5 mM) on Rb^+ uptake in human erythrocytes at 5 and 50 mM extracellular Rb^+ (mean values ± 1 SD from six paired experiments on erythrocytes of donor 1, 0.2 mM ouabain, pH 7.4, 37°C)[a]

	Na_o^+ = 0 mM			Na_o^+ = 100 mM		
	Control	Furosemide	Furosemide-sensitive	Control	Furosemide	Furosemide-sensitive
Rb_o^+ = 5 mM	0.334 ± 0.017	0.186 ± 0.015	0.148 ± 0.016	0.691 ± 0.021	0.146 ± 0.002	0.545 ± 0.026
Change due to Na_o^+				+0.357 ± 0.013[b]	−0.040 ± 0.004[c]	+0.397 ± 0.014[d]
Rb_o^+ = 50 mM	1.50 ± 0.05	1.10 ± 0.07	0.41 ± 0.07	1.74 ± 0.04	0.95 ± 0.08	0.79 ± 0.09
Change due to Na_o^+				+0.24 ± 0.04[b]	−0.15 ± 0.02[c]	+0.39 ± 0.04[d]

[a] Transport rates are given in μmol · (ml cells · hr)$^{-1}$. The principal anion was chloride. Isotonicity was maintained by choline.
[b] Acceleration of Rb^+ uptake by Na_o^+.
[c] Reduction of FR Rb^+ uptake by Na_o^+.
[d] Acceleration of FS Rb^+ uptake by Na_o^+.

FS K^+ outward transport plays a potential role in the establishment of the steady-state cation content and volume of human erythrocytes in vivo [2, 16]. Accordingly, volume changes affect the transport system, and, conversely, different activities of FS K^+ transport seem to regulate cell volume. Recently, evidence has been obtained that both 1 : 1 Na^+-K^+ cotransport and Na_o^+-independent 1 : 1 K_o^+/K_i^+ exchange activated by Na_i^+ occur in human erythrocytes [5].

In this paper, studies are presented which are designed to further characterize the properties of FS, chloride-dependent K^+ movements in human erythrocytes occurring in the absence of Na_o^+ (Na_o^+-independent) and that are additionally seen in the presence of Na_o^+ (Na_o^+-dependent), respectively. The kinetic characteristics and the dependencies on pH and cell volume of the two components are examined as well as the permissive role of Na_i^+ in Na_o^+-independent FS Rb^+ uptake. In addition, the relation of the K^+ transport pathway chemically induced by NEM to the two components of FS K^+ movements is investigated. Preliminary accounts of this work have been presented to the German and American Physiological Societies [12, 17].

Materials and Methods

Blood of apparently healthy male donors was drawn into heparin. Hematocrit and hemoglobin content of whole blood were determined. Plasma and buffy coat were removed after centrifugation (3 min, 4500 × g, room temperature). The erythrocytes were washed three times in 10 volumes of 145 mM choline chloride and immediately subjected to the transport assay or pretreated as described below. The washing and incubation solutions (300 to 315 mosmol · (kg H_2O)$^{-1}$ if not otherwise stated) contained 5 mM glucose, 1 mM phosphoric acid, 10 mM morpholinopropane sulfonic acid, titrated to the desired pH value (pH 7.4 in most cases) at 37°C with Tris-(hydroxymethyl)-aminomethane.

All transport studies were done with the Na^+-K^+ pump being inhibited by 0.2 mM ouabain. Uptake of Rb^+ was generally initiated by adding 250 μl washed erythrocytes to 25-ml incubation medium (37°C, pH 7.4). The final hematocrit was 1% or less in all experiments, and the incubation time was 1 hr in most cases. If the incubation was extended over 4 hr, the media contained 100 IU penicillin plus 30 μg streptomycin · ml^{-1}. Furosemide was dissolved in dimethylsulfoxide, the same DMSO concentration being present in the control assays (25 μl · 25 ml^{-1}). The final furosemide concentration was generally 0.5 mM.

Red cell cation contents were altered by a modification of the nystatin technique described by Cass and Dalmark [6]. 4.5-ml cells were washed three times and suspended in 25-ml dialysis media containing a total of 145 mM Na^+ + K^+ + Rb^+ (chloride salts) in varying proportions and 33 mM sucrose. The cell suspensions were gassed with CO_2 until the pH was 6.8 (room temperature). After centrifugation 1.5-ml red cell aliquots were incubated in an ice bath four times for 10 min in 12-ml dialysis medium additionally containing 25 μg nystatin per ml (200 μg nystatin per ml cells). In some experiments the concentration of Na^+ plus K^+ in the dialysis media was varied between 90 and 260 mM, resulting in mean cellular hemoglobin contents (MCHC) between 4 and 6 μmol hemoglobin tetramer · ml^{-1} when the cells were suspended in isotonic incubation media (isosmotic volume change). Subsequently, the cells were subjected to a further incubation for 10 min at 37°C and washed twice at room temperature with dialysis medium containing 0.2% bovine albumin to remove the nystatin. To obtain red cell Na^+ and K^+ contents below 0.5 μmol · ml^{-1} it was essential to remove contaminant Na^+ and K^+ from the albumin by prior dialysis. The cells were then washed three times at room temperature with 145 mM choline chloride (10 mM MOPS-Tris, pH 7.4 at 37°C, 5 mM glucose, 1 mM phosphate) and suspended in this solution at a hematocrit of close to 50% for determination of hematocrit, hemoglobin, MCHC and initial cation contents, respectively. Fluxes were initiated by adding 350 to 500 μl of the 50% cell suspensions to 25 ml prewarmed (37°C) incubation medium.

In some experiments the principal anion was nitrate. To remove Cl^- the cells were preincubated twice for 15 min at 37°C in nitrate media (hematocrit 1%) and washed twice in the same media. Choline nitrate was prepared from choline hydroxide and HNO_3.

The flux period was terminated by transferring the suspensions into an ice bath and centrifugation at 4°C. The cells were then washed three times with a 10- to 20-fold excess of ice-cold isotonic choline and sedimented by 2-min centrifugation (14,000

Table 2. Effect of external cations and furosemide (0.5 mM) on Rb uptake in 150 mM chloride media (0.2 mM ouabain, 37°C, pH 7.4)[a]

Cations	Control	Furosemide	Furosemide-sensitive
Rb^+ (50) Choline (100)	1.60 ± 0.07	1.14 ± 0.14	0.46 ± 0.07
Rb^+ (50) K^+ (100)	1.23 ± 0.11	1.01 ± 0.14	0.22 ± 0.04
Change due to K^+	−0.37 ± 0.08	−0.13 ± 0.06	−0.24 ± 0.03
Rb^+ (50) Na^+ (100)	1.84 ± 0.04	0.98 ± 0.07	0.87 ± 0.04
Change due to Na^+	+0.24 ± 0.03	−0.16 ± 0.07	+0.40 ± 0.04
Rb^+ (50) Li^+ (100)	1.62 ± 0.05	0.93 ± 0.03	0.69 ± 0.04
Change due to Li^+	+0.02 ± 0.01	−0.21 ± 0.13	+0.23 ± 0.03
Rb^+ (50) Mg^{2+} (50)-Sucrose	1.04 ± 0.05	0.75 ± 0.04	0.29 ± 0.01
Change due to Mg^{2+}	−0.56 ± 0.02	−0.39 ± 0.10	−0.17 ± 0.08
Rb^+ (150)	3.23 ± 0.21	2.73 ± 0.18	0.52 ± 0.06

[a] Numbers in parentheses: mM. Mean values ± 1 SD from four experiments with red cells of donor 3. Rb^+ uptake is given in μmol · (ml cells · hr)$^{-1}$. The changes are calculated relative to choline.

× *g*) at 4°C. Approximately 80 μl of cells were lysed with 1.6 ml of distilled water containing 6% 1-butanol (vol/vol). In the lysates, hemoglobin was measured (cyanmethemoglobin method) and Li^+, Na^+, K^+ and Rb^+ contents were determined after suitable dilution with 6% 1-butanol by atomic absorption spectrophotometry (Perkin Elmer 400), standards being prepared in the same solvent. All measurements were done in duplicate or quadruplicate with intra-assay variation coefficients of less than 1.5% for cations and less than 0.4% for hemoglobin. Essential for the reproducibility was the use of a pipettor-dilutor 1500 (CAVRO Scientific Instruments, Mtn. View, Calif.).

All red cell cation contents and transport rates given in results refer to the normal mean hemoglobin content of human erythrocytes (5.2 μmol hemoglobin tetramer or 0.335 g per ml), corresponding to a water content of 65% (weight/weight). Statistical analyses were performed by paired and unpaired Student's *t*-test where appropriate. *P* values > 0.05 were considered to be not significant.

Results

Two Components of FS Rb^+ Uptake in Na^+ Media

The effects of furosemide on ouabain-resistant Rb^+ uptake by human erythrocytes in choline and Na^+ media at 5 and 50 mM external Rb^+ (Rb_o^+) are compared in Table 1. Obviously, there is an inhibition of Rb^+ uptake by furosemide in the absence of Na_o^+. Such a *Na_o^+-independent FS Rb^+ (K^+) uptake* has been first described by Wiley and Cooper [44] and confirmed by other authors [5, 8, 21, 30, 43]. In Na^+ media FS Rb^+ uptake was accelerated due to activation of Na^+-K^+ cotransport [44], as expected. This acceleration was 3.7-fold at 5 mM and 1.9-fold at 50 mM Rb_o^+. The contribution of FS Rb^+ uptake in choline media to total FS Rb^+ uptake in the 100-mM Na^+ media was 27 and 52% at 5 and 50 mM Rb_o^+, respectively.

After replacement of chloride by nitrate the rates of FS Rb^+ uptake were decimated to less than 8% of the values given in Table 1 (*data not shown*).

Replacement of extracellular choline by Na^+ significantly ($P < 0.001$) reduced Rb^+ uptake in the presence of furosemide. The reduction of furosemide-resistant (FR) Rb^+ uptake by Na_o^+ was seen without exception in each of the experiments listed in Table 1 ($n = 6$, values indicated by superscript c) and in each experiment of the type presented in Fig. 1 ($n = 18$), Fig. 2 ($n = 8$), Fig. 4 ($n = 14$) and Fig. 6 ($n = 11$). Accordingly, the acceleration of Rb^+ uptake induced by the replacement of choline by Na_o^+ in the absence of furosemide (indicated by superscript b in Table 1) does not fully reflect the action of Na_o^+ on Rb^+ uptake by the FS transport system. The entire effect of Na_o^+ on Rb^+ uptake by the FS transport can only be assessed by the difference between the FS Rb^+ uptake rates determined in the presence and absence of Na_o^+. The enhancement of FS Rb^+ uptake by Na_o^+ (*Na_o^+-dependent FS Rb^+ uptake*) is indicated by superscript d in Table 1. Obviously Na_o^+-dependent FS Rb^+ uptake is significantly greater than the Rb^+ uptake induced by Na_o^+ in the absence of furosemide. The difference is due to the lowering of FR Rb^+ uptake by Na_o^+ described above.

Furosemide-resistant (FR) Rb^+ uptake was reduced after replacement of external choline by other cations with increasing efficiency in the order $K^+ < Na^+ < Li^+ < Mg^{2+}$ (Table 2). In the absence of furosemide, the only cation that accelerated Rb^+ uptake was Na^+; Li^+ had a negligible effect and K^+ and Mg^{2+} were inhibitory (Table 2).

FS Rb^+ uptake was enhanced by Na_o^+, and to a lesser extent by Li_o^+, and reduced by K_o^+ and Mg_o^{2+}. Accordingly, Li_o^+ is capable of replacing Na_o^+ in pro-

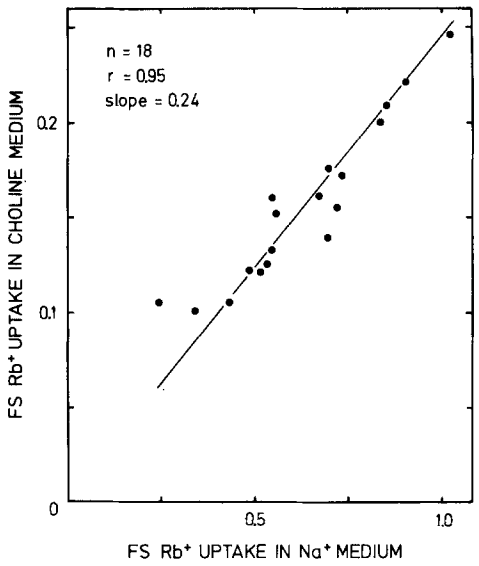

Fig. 1. Correlation between FS Rb^+ uptake (μmol · (ml cells · hr)$^{-1}$) determined in 145 mM Na^+ media (abscissa) and 145 mM choline media (ordinate) in red cells of 18 apparently healthy donors. The Rb^+ concentration was 5 mM, and the principal anion was chloride (0.2 mM ouabain, 37°C, pH 7.4). Furosemide-resistant Rb^+ uptake was 0.125 ± 0.020 in the Na^+ media and 0.175 ± 0.019 (± SD) μmol · (ml cells · hr)$^{-1}$ in choline media in the 18 donors

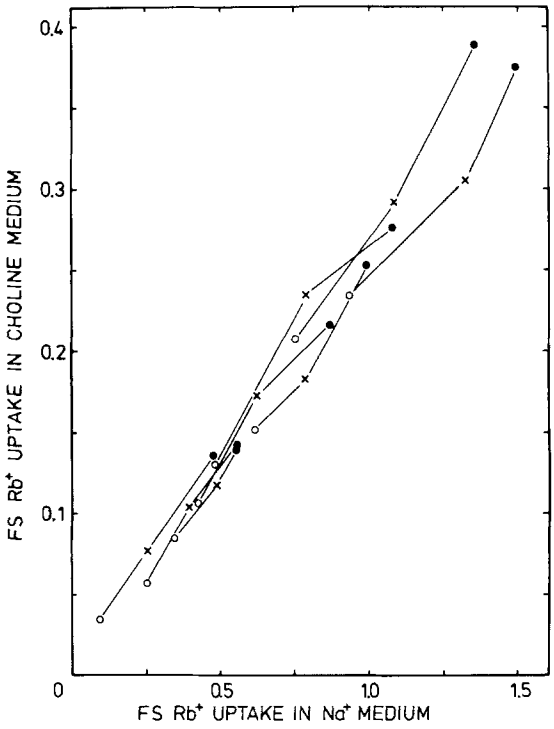

Fig. 2. Relation between FS Rb^+ uptake in 135 mM NaCl media and FS Rb^+ uptake in 135 mM choline media in young (○), unseparated (×) and old (●) erythrocytes of eight donors with differing activities of the FS transport system (5 mM Rb_o^+, 37°C, pH 7.4, 0.2 mM ouabain). The data of the individual donors are connected by lines. Transport rates (μmol · (ml cells · hr)$^{-1}$) are normalized to the MCHC of 5.2 μmol Hb_4 · (ml cells)$^{-1}$. The mean MCHC values (μmol Hb_4 · (ml cells)$^{-1}$ ± SD) were 5.20 ± 0.21 in the unseparated and 4.98 ± 0.13 in the lighter (younger) and 5.48 ± 0.18 in denser (older) cells, respectively. The cells were washed three times (room temperature) in a 5-mM K^+ − 130 mM Na^+ − 10 mM MOPS-Tris buffer (5 mM glucose, 1 mM inorganic phosphate, 290 to 293 mosmol · (kg H_2O)$^{-1}$, pH 7.40 at 37°C) and divided into two parts. After 15 min of centrifugation (4500 × *g*) at 12°C, the two upper and lower parts of the packed cells were combined, resuspended in the 5-mM K^+ − 130 mM Na^+ medium, and the 15-min centrifugation at 12°C was repeated. MCHC and transport rates were measured on the upper half of the lighter (young) and the lower half of the denser (old) cells obtained after the second centrifugation. Reticulocyte counts were 9.6‰ in the unseparated and 23.4 and 2.9‰ in the young and old cells, respectively

moting FS Rb^+ uptake, at least in part [4, 11]. FS Li^+ uptake (0.27 ± 0.09 μmol · (ml cells · hr)$^{-1}$) was similar to the Li_o^+-dependent component of FS Rb^+ uptake listed in Table 2, thus suggesting a 1 : 1 Li^+-Rb^+ inward cotransport. The reduction of FS Rb^+ uptake by K_o^+ and Mg_o^{2+} can be explained by the competition of K_o^+ and Rb_o^+ for the transport site [14] and the inhibition of the transport system by Mg_o^{2+} [11, 22]. In pure Rb^+ media there was a substantial FS Rb^+ uptake (Table 2), indicating that the FS Rb^+ transport occurring in the absence of Na_o^+ (or Li_o^+) does not require the cations choline$^+$, K^+ or Mg^{2+} replacing Na_o^+.

From the data presented in Tables 1 and 2 it is concluded that FS Rb^+ uptake in Na^+ media exhibits two components: *i*) the first is independent of Na_o^+ (*Na_o^+-independent FS Rb^+ uptake*) and also seen in choline, K^+, Mg^{2+} and pure Rb^+ media, *ii*) and the second requires the presence of Na_o^+ (or Li_o^+) (*Na_o^+-dependent FS Rb^+ uptake*).

Interindividual and Red Cell Age-Dependent Differences of the Two Components

FS Rb^+ uptake at 5 mM Rb_o^+ in 145 mM Na^+ and choline media was compared in 18 donors with individual different activities of the transport system (Fig. 1). Obviously, there exists a highly significant positive correlation ($P < 0.001$) between FS Rb^+ uptake in Na^+ and choline media. The slope of the relation in Fig. 1 is 0.244, indicating that Na_o^+-independent FS Rb^+ uptake in choline media comprises 24.4% of total FS Rb^+ uptake in Na^+ media at 5 mM Rb_o^+, independent of the individual activity of the transport system. The remaining 75.6% of FS Rb^+ uptake in Na^+ media are mediated by the Na_o^+-dependent component of FS Rb^+ uptake.

The rates of FS Rb^+ uptake in choline, in Na^+ media and the Na_o^+-dependent component were not significantly related to the red cell Na^+ content (*data not shown*).

The two components of FS Rb^+ uptake were studied in cells separated according to age (density) by centrifugation on eight further donors. In Fig. 2, the data obtained on the cell fractions of the individual donors are connected by lines. Obviously, Na_o^+-independent FS Rb^+ uptake in choline media rose in parallel to total FS Rb^+ uptake in Na^+ media by 1.6 to fivefold in each of the eight cell specimens when going from the young to the unseparated to the old cells, independent of the individual activity of the transport system of the donors (Fig. 2). The age-dependent changes of FS Rb^+ uptake in Na^+ media reported in Fig. 2 are in accordance with recent results of Chipperfield and Mangat [9]. The extremes of the transport rates seen in the slowest young cell population and the fastest old cell populations varied by a factor of greater than 10.

The mean values of the FS transport rates of the donors examined in Fig. 2 were lowered by about 25% in the younger and accelerated by about 30% in the older cells as compared to the unseparated erythrocytes, both in Na^+ and in choline media. The percentage contribution of Na_o^+-independent FS Rb^+ uptake in choline media to total FS Rb^+ uptake in Na^+ media (25.8 ± 2.2% (SD) in unseparated cells) was not significantly altered in the young (26.7 ± 4.3%) and the old erythrocytes (26.2 ± 1.6%).

The data compiled in Figs. 1 and 2 demonstrate that the *Na_o^+-independent component of FS Rb^+ uptake in choline media and the Na_o^+-dependent component of FS Rb^+ uptake additionally present in Na^+ media increase strictly in parallel to each other* both in erythrocytes of different donors with varying activities of the FS transport system and in erythrocytes ageing in vivo within individual donors.

Sensitivity of the Two Components to Inhibition by Furosemide and Bumetanide

Increasing the furosemide concentration above 0.5 mM or using different concentrations of bumetanide produced no greater inhibition of Rb^+ uptake than that obtained with 0.5 mM furosemide, both in choline and in Na^+ media (Fig. 3). Rather, the drug-sensitive uptake fell at concentrations above 0.5 mM furosemide and bumetanide due to an increase of the Rb^+ leak.

The Na_o^+-dependent component was much more sensitive to the loop diuretics than the Na_o^+-independent one. The slope of the dose-effect relationship was steeper in Na^+ than in choline media.

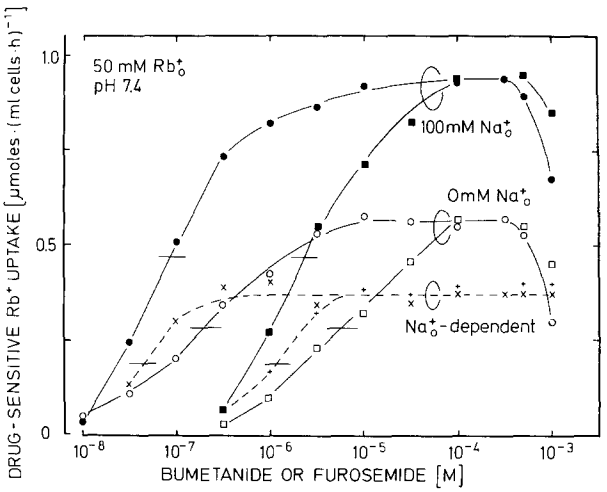

Fig. 3. Drug-sensitive Rb^+ uptake as a function of bumetanide (●, ○) and furosemide (■, □) concentration determined at 50 mM Rb_o^+ in 100 mM Na^+ media (●, ■) or 100 mM choline media (○, □) (pH 7.4, 37°C, 0.2 mM ouabain). Na_o^+-dependent uptake (× due to bumetanide, + due to furosemide) is the difference between drug-sensitive uptake rates determined in Na^+ and choline media. Similar results were obtained on red cells of three other donors and at pH 8.0

Half-maximum inhibition of the Na_o^+-dependent component was observed at about 4.6×10^{-8} M bumetanide and 1.1×10^{-6} M furosemide, and for the Na_o^+-independent component at about 1.9×10^{-7} M bumetanide and 5.6×10^{-6} M furosemide, respectively. Half-maximum inhibition of total Rb^+ uptake in 100 mM Na^+ media occurred at intermediate inhibitor concentrations (about 8.7×10^{-8} M bumetanide and 2.2×10^{-6} M furosemide, respectively; *see* Fig. 3).

Kinetic Properties and pH Dependence

The dependencies of Rb^+ uptake on Rb_o^+ in 100 mM choline and Na^+ media are compared in Fig. 4. In the presence of furosemide, Rb^+ uptake was not a linear function of Rb_o^+ but comprised a small saturating component, both in choline and in Na^+ media. This nonlinearity is possibly due to a reduction of the FR Rb^+ uptake rate constant occurring upon replacement of choline by Rb_o^+, in analogy to the reduction of the Rb^+ leak caused by replacement of choline by K_o^+, Na_o^+, Li_o^+ and Mg_o^{2+} in Tables 1 and 2 (*see* Discussion).

The contribution of Na_o^+-independent FS Rb^+ uptake in choline media to total Rb^+ uptake in the 100 mM Na^+ media in Fig. 4 rose from 20% at 2 mM Rb_o^+ to 27, 34, 39 and 52% upon increasing Rb_o^+ to 5, 10, 20 and 50 mM, respectively.

The effect of pH changes on Rb^+ uptake in Na^+ and choline media is examined in Fig. 5. At Rb^+ concentrations below 50 mM FR Rb^+ uptake (closed symbols) was always higher in choline than in Na^+ media, and was lower at pH 7.4 than at pH 6.7 and 8.0, respectively.

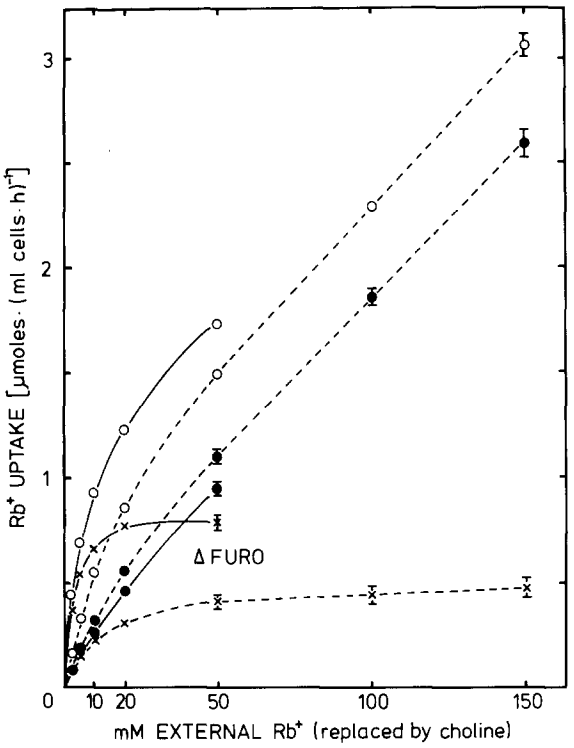

Fig. 4. Rb^+ uptake as a function of Rb_o^+ in choline (broken lines) and 100 mM Na^+ media (solid lines). ○ = control, ● = 0.5 mM furosemide, × = FS (0.2 mM ouabain, 37°C, pH 7.4). Mean values ± 1 SEM (indicated by brackets) of seven experiments with red cells of donor 3 with a cell Na^+ content of 5.5 μmol · ml^{-1}

Rb^+ uptake at the fixed Rb^+ concentration of 5 mM is studied as a function of Na_o^+ at the three pH values in Fig. 6. The reduction of FR Rb^+ uptake by Na_o^+ was most pronounced at pH 8.0.

Na_o^+-dependent FS Rb^+ uptake is related to Rb_o^+ at the three pH values in Fig. 7. Obviously, the transport rates increase with rising pH values. Maxima of Na_o^+-dependent FS Rb^+ uptake are seen at about 10, 15 and 25 mM Rb_o^+ at pH 6.7, 7.4 and 8.0, respectively. The fall of the transport rates occurring at higher Rb^+ concentrations is most pronounced at pH 6.7. Such a fall of the reaction rate at high substrate concentrations is well-known to enzyme kineticists as the phenomenon of substrate inhibition [10]. In the case of linear substrate inhibition the reaction rate V is described by Eq. (1):

$$V = \frac{V_{max}}{1 + K_m/s + s/K_i} \quad (1)$$

in which V_{max}, K_m, K_i and s are the apparent maximum velocity, the apparent Michaelis-Menten and inhibition constants, and the substrate concentration, respectively [10]. The curves in Fig. 7 are the best least-squares fits of the data to Eq. (1). The kinetic constants are listed in Table 3. With the same fitting procedure, a significant substrate inhibition was also observed for total FS Na^+ uptake in Na^+ media (Table 3). The apparent K_i values obtained both for FS Rb^+ uptake in choline media and for Na_o^+-dependent FS Rb^+ uptake as a function of Na_o^+ at the fixed concentration of 5 mM Rb_o^+, however, exceeded 10^6 mM, indicating that neither Rb_o^+ in choline media nor Na_o^+ produce significant sub-

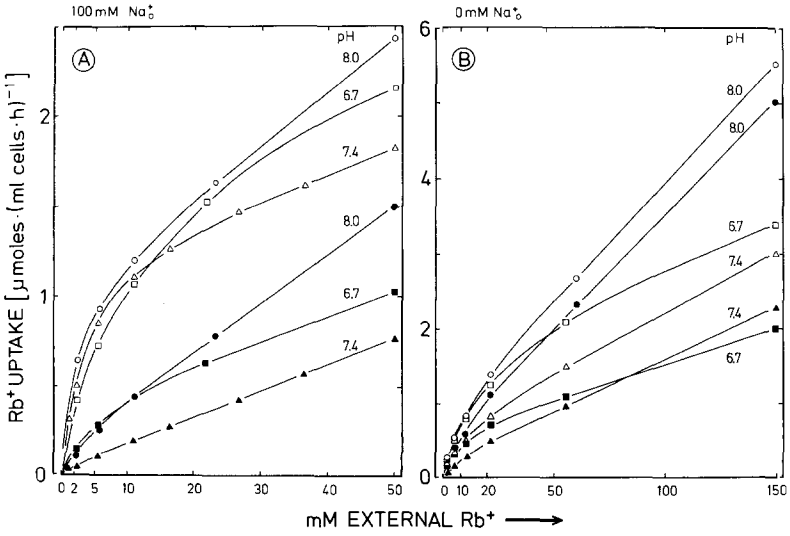

Fig. 5. Dependence of Rb^+ uptake on Rb_o^+ in 100 mM Na^+ (*A*) and 100 mM choline media (*B*) at pH 6.7, 7.4 and 8.0. The principal anion was chloride; isotonicity was maintained with choline. Open symbols: controls, filled symbols: 0.5 mM furosemide (0.2 mM ouabain, 37°C). Single experiment with red cells of donor 3. Note the differences in the scales of the abscissa and ordinate in panels (*A*) and (*B*)

strate inhibition in the concentration range examined. Accordingly, the data of the latter experiments were analyzed assuming simple Michaelis-Menten kinetic using the Eadie plot. The results obtained were very similar to those found by fitting the data to Eq. (1).

The apparent kinetic constants collected are compiled in Table 3. Increasing the pH from 6.7 to 8.0 reduced the apparent K_m Rb_o^+ of Na_o^+-independent FS Rb^+ uptake in choline media from 45 to 10.5 mM, and lowered its apparent maximum velocity. In 100 mM Na^+ media, the apparent $K_m Rb_o^+$ decreased from 11 to 2.7 mM with rising pH, whereas the apparent maximum velocity remained almost unaffected.

Na_o^+-dependent FS Rb^+ uptake exhibited a much higher affinity for Rb_o^+ with apparent K_m values close to 2 mM. It showed a twofold rise in the

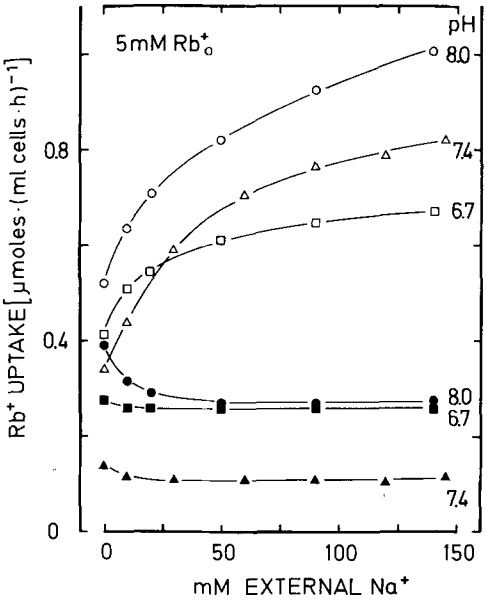

Fig. 6. Dependence of Rb^+ uptake on Na_o^+ at a fixed Rb^+ concentration of 5 mM and pH values of 6.7, 7.4 and 8.0. The principal anion was chloride; isotonicity was maintained with choline (0.2 mM ouabain, 37°C). Open symbols: controls; filled symbols: 0.5 mM furosemide. Single experiment with red cells of donor 3

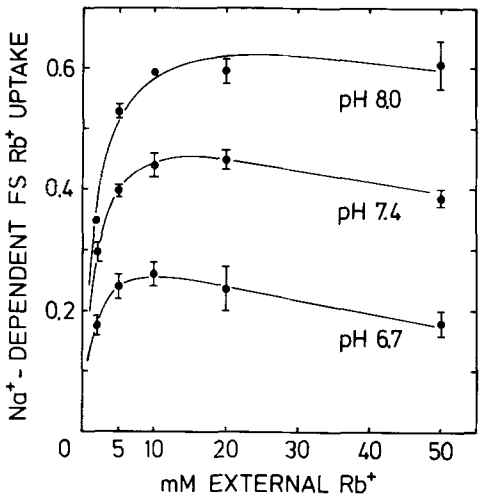

Fig. 7. Na_o^+-dependent FS Rb^+ uptake (μmol · (ml cells · hr)$^{-1}$) induced by replacement of 100 mM choline Cl by 100 mM NaCl as a function of Rb_o^+. Isotonicity was maintained by choline Cl. Mean values (± 1 SEM, indicated by bars) of four experiments at pH 6.7, 7 experiments at pH 7.4 and 3 experiments at pH 8.0 with erythrocytes of donor 3. The curves are the best least-squares fits of the data to Eq. (1)

Table 3. Apparent kinetic constants of Na_o^+-independent and Na_o^+-dependent FS Rb^+ uptake determined in experiments similar to those shown in Figs. 4–7 with red cells of donor 3[a]

	In choline (Na^+-independent)		In 100 mM Na^+			Na_o^+-dependent				Na_o^+-dependent[b]	
	K_{mRb^+}	V_{max}	K_{mRb^+}	V_{max}	K_{iRb^+}	K_{mRb^+}	V_{max}	K_{iRb^+}		K_{mNa^+}	V_{max}
pH 6.7 (n = 4)	44.8 ± 9.1	1.55 ±0.37	11.4	1.19	>10^3	2.04	0.36	51	pH 6.6 (n = 2)	26.5	0.34
pH 7.4 (n = 7)	12.4 ± 3.1	0.51 ±0.10	2.93	0.88	903	1.80	0.56	129	pH 7.4 (n = 6)	25.7 ±6.1	0.61 ±0.06
pH 8.0 (n = 3)	10.5 ± 1.4	0.44 ±0.04	2.66	1.01	472	1.91	0.71	332	pH 8.0 (n = 3)	29.2 ±6.0	0.71 ±0.09

[a] Similar results were obtained in experiments with red cells of two other donors. Number of experiments are given in parentheses. K_m and K_i values are in mM, V_{max} values in μmol Rb^+ uptake · (ml cells · hr)$^{-1}$. The data were either calculated from the individual experiments using the Eadie plot and mean values ± 1 SD are given, or they were obtained from the best least-squares fits of the mean values to Eq. (1) as described in the text.

[b] Extracellular Rb^+ concentration fixed at 5 mM.

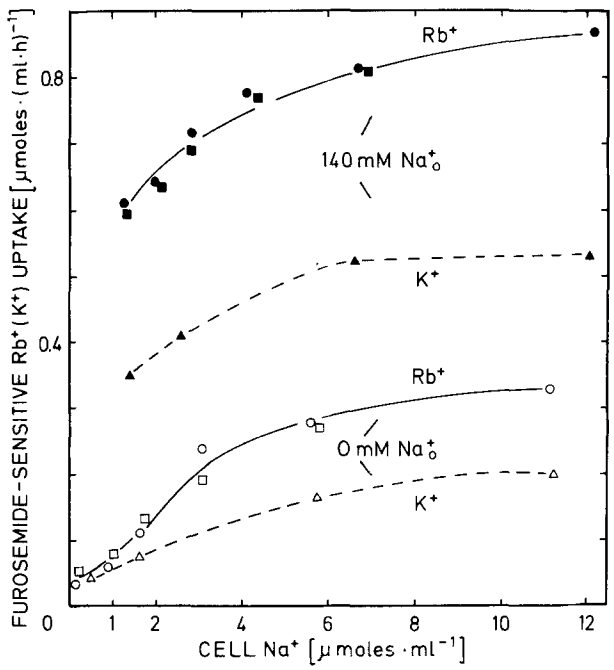

Fig. 8. Effect of cell Na^+ content on Na_o^+-independent FS Rb^+ uptake (solid lines, indicted by Rb^+) and K^+ uptake (broken lines, indicated by K^+) in choline media (open symbols) and on total Rb^+ and K^+ uptake in Na^+ media (filled symbols) (10 mM Rb_o^+ or K_o^+, pH 7.4, 37°C, 0.2 mM ouabain). Cell cation contents were varied by using nystatin (*see* Materials and Methods). The cell Na^+ given on the abscissa is the arithmetic mean of the values determined before and after termination of the 1-hr influx period. The filled and open circles and squares refer to two separate paired experiments on cells of donor 1, the filled and open triangles to cells of the same donor in which cell K^+ was replaced by Rb^+ (residual $K^+ < 0.5$ μmol · (ml cells)$^{-1}$)

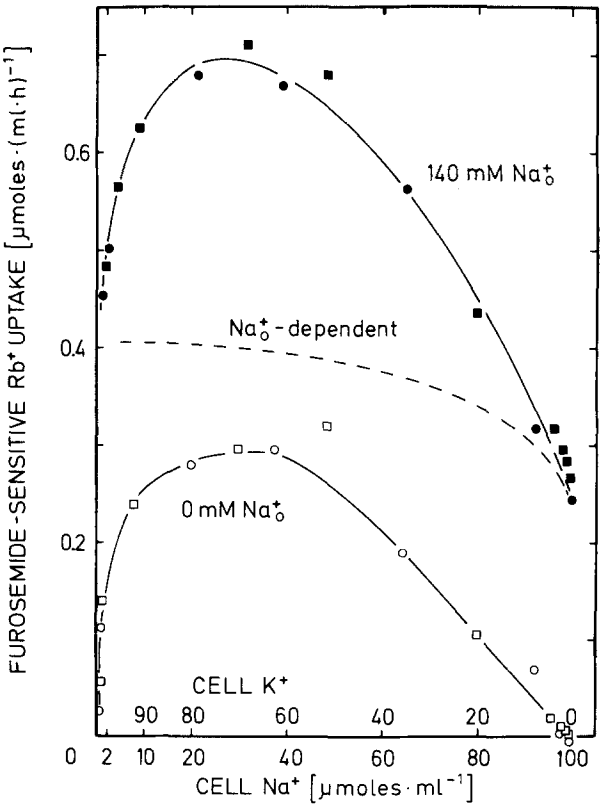

Fig. 9. Effect of the red cell Na^+ and K^+ content on FS Rb^+ uptake in choline and Na^+ media (10 mM Rb_o^+, 37°C, pH 7.4, 0.2 mM ouabain). The broken curve (Na_o^+-dependent component) is the difference between the two other curves. Cell Na^+ and K^+ contents were varied at the expense of each other using nystatin. The cell cation contents given on the abscissa are the arithmetic means of the values determined before and after the 1-hr influx period. The data are collected from two experiments (circles and squares) on erythrocytes of donor 2. Similar results were obtained with erythrocytes of four other donors

maximum velocity upon elevation of the pH, in contrast to the Na_o^+-independent component in choline media which is slowed down to one-third at high pH values (Table 3). The maximum rates of the two modes of Rb^+ uptake by the FS transport system are identical at a pH value of close to 7.4.

The substrate inhibition of Na_o^+-dependent FS Rb^+ uptake by Rb_o^+ was most pronounced at low pH, the apparent K_i value increasing from 51 mM Rb_o^+ at pH 6.7 to 332 mM Rb_o^+ at pH 8. The apparent inhibitory constants obtained for total FS Rb^+ uptake in Na^+ media were higher, and they showed the reverse pH dependence. The latter observation may be related to the increased contribution of Na_o^+-dependent to total FS Rb^+ uptake in Na^+ media at high pH values.

The last two rows of Table 3 show kinetic constants for the activation by Na_o^+ of FS Rb^+ uptake at the fixed concentration of 5 mM Rb_o^+. The apparent $K_m Na_o^+$ of Na_o^+-dependent FS Rb^+ uptake was close to 25 mM and almost independent of the pH. The maximum velocity of this component increased with rising pH, in concordance with the results obtained at varying Rb^+ concentrations.

The sums of the apparent V_{max} values obtained for Na_o^+-independent and Na_o^+-dependent FS Rb^+ uptake exceed the apparent V_{max} values seen in Na^+ media, especially at pH 6.7 where the Na_o^+-independent component predominates (Table 3). One possible explanation of this phenomenon is that Na_o^+ causes an inhibition of the Na_o^+-independent component which, however, was not detected because FS Rb^+ uptake was not studied at Na^+ concentrations exceeding 145 mM.

It is well known that erythrocytes shrink and swell at alkaline and acid pH values, respectively. The question thus arises as to whether volume changes contribute to the pH-dependent alterations of the kinetic properties of the FS—transport system described in Table 3. Although this question cannot be definitely answered at present, it appears that the 6.4% increase in cell volume at pH 6.7 and the 5.5% decrease at pH 8.0 relative to pH 7.4 [25] are much too small as to substantially alter the two components of FS Rb^+ uptake (*see* Fig. 12). The volume changes

correspond to a decrease and increase of the MCHC from 5.2 at pH 7.4 to 4.89 and 5.50 μmol Hb_4 · (ml cells)$^{-1}$ at pH 6.7 and 8.0, respectively.

Effects of Intracellular Na^+ and K^+

The two components of FS Rb^+ (K^+) uptake are studied in Figs. 8 and 9 on cells containing various concentrations of Na^+, K^+ and Rb^+ after treatment with nystatin. The two solid curves in Fig. 8 relate FS Rb^+ uptake in choline and 140 mM Na^+ media to the red cell Na^+ content (Na_i^+) in the range between 0.1 and 12 μmol Na^+ · ml^{-1}. The Na_o^+-independent component gradually disappeared upon lowering Na_i^+, the relation of the uptake to Na_i^+ being sigmoidal. Half-maximum transport rates were achieved in the range of 2 to 3 μmol Na^+ per ml cells. In 140 mM Na^+ media there was an increase by about 40% of FS Rb^+ uptake upon elevation of Na_i^+ from 1.5 to 12 μmol · (ml cells)$^{-1}$. The Na_o^+-dependent component of FS Rb^+ uptake (the difference between the two solid curves in Fig. 8) was almost independent of Na_i^+ in the range studied in Fig. 8. Similar results were obtained in experiments in which the red cell Na^+ content was varied by a 24-hr preincubation at 37°C in media of differing cation composition without and with added ouabain (*data not shown*).

The two broken curves in Fig. 8 depict FS K^+ uptake by cells in which internal K^+ was replaced by Rb^+. K^+ uptake by the Rb^+ cells was also accelerated by Na_i^+, but was lower than the Rb^+ uptake in high K^+ cells. The sigmoidal dependence on Na_i^+ of Rb^+ uptake seen in high K^+ cells was not observed for K^+ uptake by Rb^+ cells. In Na^+ media, K^+ uptake was substantially enhanced in the Rb^+ cells.

In the experiments of Fig. 9 red cell Na^+ and K^+ were varied between 0.1 and 100 μmol · ml^{-1} at the expense of each other. The Na_o^+-independent FS Rb^+ uptake in choline media displayed the increase with rising Na_i^+ at low Na^+ contents already described in Fig. 8. A maximum is reached between 20 and 40 μmol Na_i^+ · (ml cells)$^{-1}$. With further elevation of Na_i^+, Na_o^+-independent uptake in choline media falls and vanishes in cells containing only Na^+ and no K^+. Accordingly, Na_o^+-independent FS Rb^+ uptake requires not only Na_i^+ but also K_i^+, Na_i^+ activating more effectively than K_i^+ with apparent K_m values close to 2 and 30 μmol · ml^{-1} Na_i^+ and K_i^+, respectively.

Total FS Rb^+ uptake in Na^+ media follows the same pattern as the Na_o^+-independent component. It exhibits the same absolute acceleration by about 0.3 μmol · (ml cells · hr)$^{-1}$ upon increasing Na_i^+ from below 0.5 to 20 to 40 μmol per ml cells, and a fall at higher values of Na_i^+. However, in Na^+ media the uptake does not drop down to zero in cells containing only Na^+ or only K^+, indicating that the Na_o^+-dependent component of FS Rb^+ uptake does not require K_i^+ or Na_i^+. The bell-shaped curve of FS Rb^+ uptake in Na^+ media in Fig. 9 is in accordance with the view that the Na_o^+-independent and Na_o^+-dependent modes of FS Rb^+ uptake largely occur additive to each other.

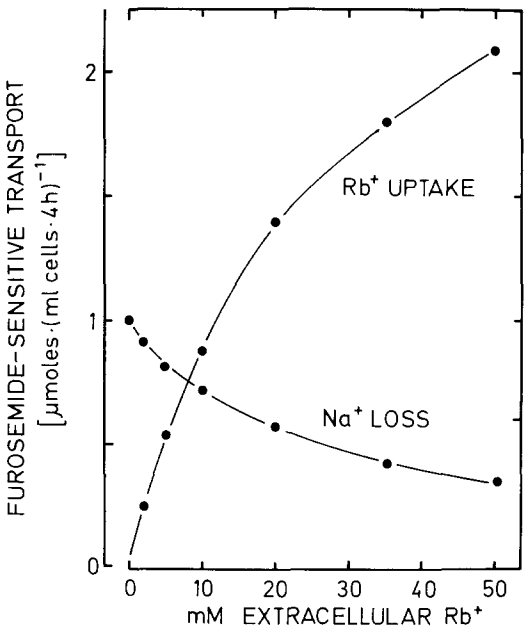

Fig. 10. FS Na^+ loss and Rb^+ uptake in choline media as a function of Rb_o^+ (extracellular choline was replaced by Rb^+, 37°C, pH 7.4, 0.2 mM ouabain). Transport rates were measured over 4-hr on cells preincubated in pure Na^+ media with ouabain to increase the cell Na^+ content to 10 μmol · ml^{-1}

The Na_o^+-dependent fraction of FS Rb^+ uptake does not vary at cell Na^+ contents up to 40 μmol · ml^{-1}. At higher Na^+ contents, the Na_o^+-dependent component tends to fall (broken curve in Fig. 9). This fall was slightly more pronounced in experiments with red cells of three other donors.

The question as to whether the activating effects of Na_i^+ and K_i^+ on Na_o^+-independent FS Rb^+ uptake are coupled to a Rb_o^+-activated efflux of these ions is examined in Figs. 10 and 11. FS Na^+ release into choline media was not accelerated but rather inhibited by Rb_o^+ (Fig. 10). Half-maximum inhibition was achieved at about 15 mM Rb_o^+, a concentration corresponding to the apparent K_m value of Rb_o^+ for Na_o^+-independent Rb^+ uptake. Accordingly, the transacceleration of Na_o^+-independent Rb^+ uptake by Na_i^+ seems not to be associated with an acceleration by Rb_o^+ of Na^+ outward net transport.

The action of Rb_o^+ on Na_o^+-independent (Na_i^+-

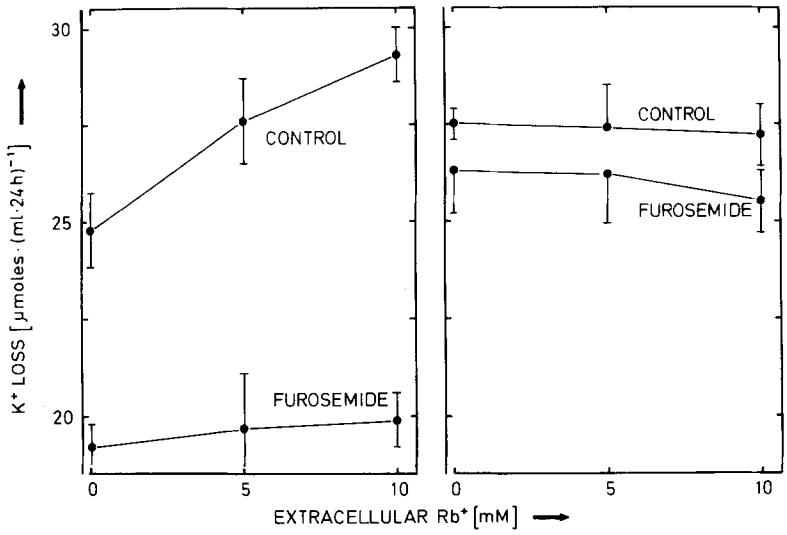

Fig. 11. Effect of Rb_o^+ on K^+ loss in the absence and presence of 0.5 mM furosemide in erythrocytes of donor 2 incubated over 24 hr in Na^+ media (*left*, $n = 6$, mean cellular Na^+ content over time 15 μmol · ml^{-1}) and in cells preincubated for 4 hr in isotonic K^+ media to lower cell Na^+ to 1.7 ± 0.2 μmol · ml^{-1} and incubated over 24 hr in choline media (*right*, $n = 3$, mean cellular Na^+ content over time 0.9 μmol · ml^{-1}) (37°C, pH 7.4, 0.2 mM ouabain). Similar results were obtained on erythrocytes of two other donors

Table 4. Estimation of the stoichiometry of Na_i^+-dependent (Na_o^+-independent) furosemide-sensitive Rb_o^+/K_i^+ exchange[a]

	0 mM Rb_o^+	5 mM Rb_o^+	10 mM Rb_o^+
a) FS Rb^+ uptake	—	9.06 ± 0.75	11.3 ± 0.66
b) Na_o^+-independent (Na_i^+-dependent) fraction of FS Rb^+ uptake	—	2.17 ± 0.19	4.52 ± 0.21
c) FS K^+ loss	5.55 ± 1.45	7.87 ± 1.15	9.42 ± 1.17
d) Acceleration of FS K^+ loss by Rb_o^+	—	2.32 ± 0.21	3.87 ± 0.29
e) b/d	—	0.94 ± 0.07	1.17 ± 0.12

[a] The data in rows a), c) and d) are from the experiments shown in the left-hand panel of Fig. 11. The values in row b) are calculated as described in the text. Transport rates in μmol · (ml · 24 hr)$^{-1}$ determined in 140 mM Na^+ media in six paired experiments on cells of donor 2. Similar results were obtained with red cells of two other donors. Mean values ± 1 SD

dependent) FS K^+ release was studied by measuring FS K^+ loss over 24 hr on fresh cells in Na^+ media and on Na^+-depleted cells in choline media. In cells containing 15 μmol Na^+ · ml^{-1}, Rb_o^+ accelerated K^+ loss in the absence but not in the presence of furosemide. This effect was absent in cells with a Na^+ content lower than 1 μmol · ml^{-1} (Fig. 11). It is concluded, therefore, that Rb_o^+ stimulates a FS K^+ loss that requires Na_i^+.

The stoichiometry of the Na_i^+-dependent FS Rb^+ uptake and K^+ loss is estimated in Table 4. Row a) gives the FS Rb^+ uptake in 140 mM Na^+ media over 24 hr at 5 and 10 mM Rb_o^+. The fractions of Na_o^+-independent FS Rb^+ uptake in row b) are calculated from row a) taking into account that about 25% of FS Rb^+ uptake in Na^+ media are dependent on Na_i^+ at 5 mM Rb_o^+ (Fig. 1), The percentage increasing to 40% at 10 mM Rb_o^+ in cells containing 15 to 20 μmol Na^+ · ml^{-1} (Fig. 9). Rows c) and d) show the dependence of FS K^+ loss on Rb_o^+. The stoichiometries of Na_i^+-dependent FS Rb^+ uptake and FS K^+ loss induced by Rb_o^+ obtained by dividing the values in row b) by those in row d) were not significantly different from 1.0 (row e) in Table 4). It is concluded, therefore, that the Na_o^+-independent FS Rb^+-K^+ transport represents a 1 : 1 Rb_o^+/K_i^+ exchange which requires Na_i^+, the internal Na^+ being not extruded. Similar properties of a FS, chloride-dependent Rb_o^+/K_i^+ exchange have been observed by other authors in duck [29, 39–41] and human erythrocytes [5].

Effect of Cell Volume

The effect of changes in cell volume on the components of FS Rb^+ uptake is investigated in Fig. 12. Two procedures were applied to alter the cell volume. In the *osmotic* method, the osmolality of the incubation media was increased stepwise from 190

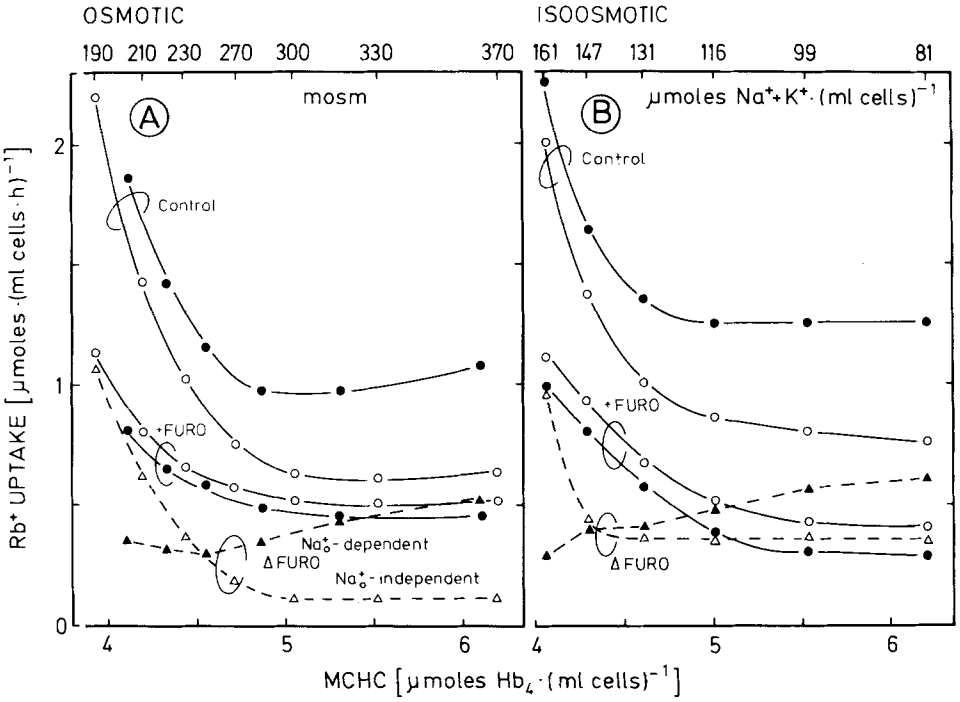

Fig. 12. Dependence of ouabain-resistant Rb^+ uptake on cell volume. Cell volume was changed by altering the medium osmolality (*osmotic method*) in panel (*A*) and by pretreatment of the cells using nystatin (*see* Materials and Methods) and suspending the cells in isotonic media (*isosmotic method*) in panel (*B*). The Rb^+ uptake rates refer to cells with a MCHC of 5.2 μmol $Hb_4 \cdot ml^{-1}$ (10 mM Rb_o^+, 37°C, pH 7.4). The incubation media contained 80 mM NaCl (●) or 80 mM choline Cl (○) plus increasing amounts of sucrose in panel (*A*) or 100 mM sucrose in panel (*B*). The osmolalities and Na^+ plus K^+ contents (referring to a MCHC of 5.2 μmol $Hb_4 \cdot ml^{-1}$) are given on the upper abscissas. In the osmotic method (*A*) fresh cells were used. In the isosmotic method (*B*) cell Na^+ was maintained at 20 to 25 mmol · (liter cell water)$^{-1}$. 0.5 mM furosemide was present where indicated. The triangles denote FS uptake in choline media (△) and the Na_o^+-dependent component (▲) additionally present in Na^+ media. Similar results were obtained in experiments on three other donors. The actual mean cellular hemoglobin content is given at the abscissa as indicator of cell volume. A lowering of the MCHC from its normal value of 5.2 to 4.5 and 4.0 μmol $Hb_4 \cdot ml^{-1}$ indicates a cell swelling from a relative volume of 1.0 to a volume of 1.16 and 1.30; conversely, as the MCHC increases to 6.0 and 6.2, the cells shrink to a relative volume of 0.87 and 0.84

to 370 mosmol · (kg $H_2O)^{-1}$ by addition of sucrose (panel *A*). In the *isosmotic* method, the cellular K^+ content was altered by using nystatin (*see* Materials and Methods) and the erythrocytes were suspended in isotonic media (panel *B*).

Similar results were obtained with the two methods. Upon cell swelling (indicated by a lowering of the actual MCHC) the total Rb^+ uptake increased gradually, both in Na^+ and choline media. 0.5 mM furosemide reduced the Rb^+ transport accelerations seen in swollen cells by about one-half. Replacement of Cl^- by NO_3^- blocked the swelling effect almost entirely (*data not shown*). The Na_o^+-independent component of FS Rb^+ uptake in choline media was accelerated 10-fold upon lowering the MCHC from 5.03 to 3.96 μmol $Hb_4 \cdot ml^{-1}$ by the osmotic method (corresponding to a cell swelling by 27%); it was not altered in shrunken cells (Fig. 12*A*). With the isosmotic method the enhancement of the Na_o^+-independent component of FS Rb^+ uptake occurred only at relative cell volumes above 1.2 (Fig. 12*B*).

The Na_o^+-dependent component of FS Rb^+ uptake was much less sensitive to cell volume changes and exhibited a slight trend for an acceleration in shrunken cells, both with the osmotic and isosmotic method (Fig. 12). It is concluded, therefore, that an acceleration of Na_o^+-independent FS Rb^+ uptake in human erythrocytes swollen by more than 20% is a characteristic property not shared by the Na_o^+-dependent component.

Effect of NEM

The possible relation of chloride-dependent Rb^+ transport chemically induced by NEM [11, 33, 35–37, 43] to Na_o^+-independent FS Rb^+ uptake occurring in normal human erythrocytes is studied in Figs. 13 and 14 and Table 5. In cells treated with

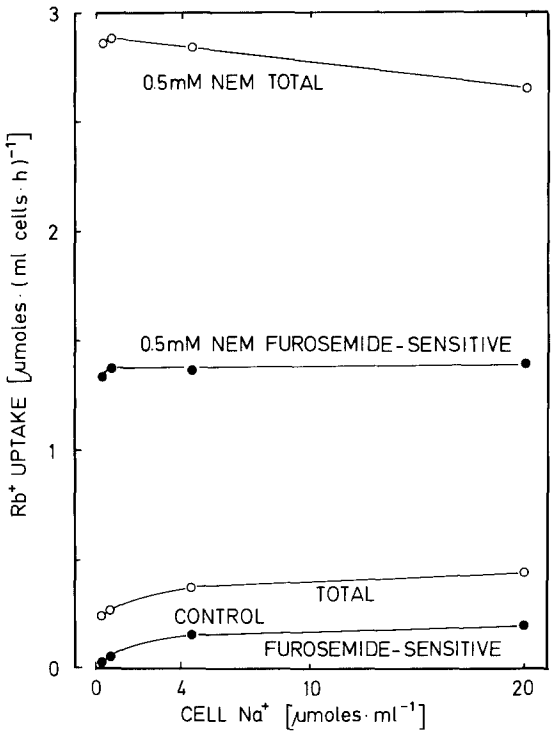

Fig. 13. Dependence of Na_o^+-independent Rb^+ uptake in choline media on cell Na^+ content in the absence (control) and presence of 0.5 mM NEM (molar ratio of NEM to Hb_1 = 2.4) and 0.5 mM furosemide, respectively (5 mM Rb_o^+, 37°C, pH 7.4, 0.2 mM ouabain). Cell Na^+ was varied by using nystatin

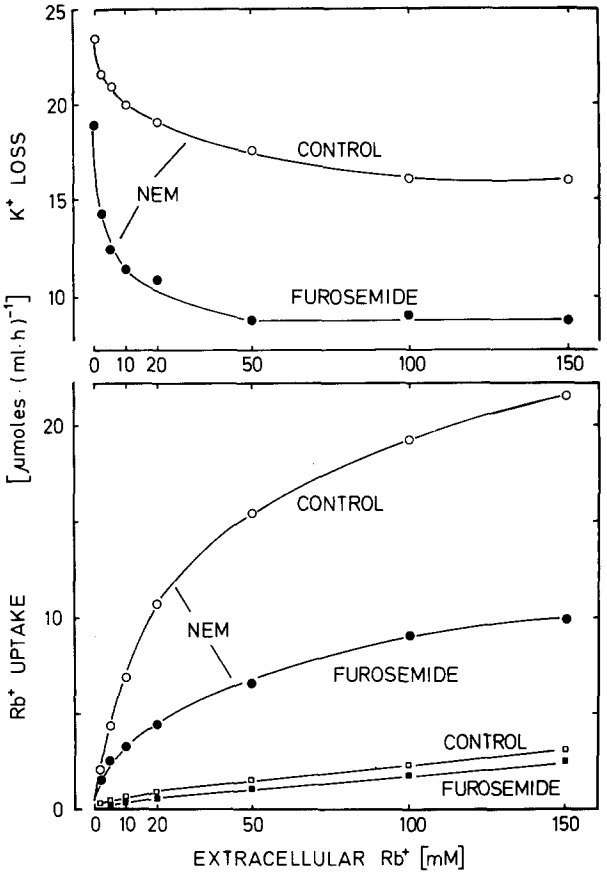

Fig. 14. Rb^+ uptake and K^+ loss in cells pretreated with NEM as a function of Rb_o^+ (choline replacement). Pretreatment with NEM as described in Table 5 (pH 7.4, 37°C, 0.2 mM ouabain)

NEM, Rb^+ uptake in choline media was increased by about 10-fold, approximately half of the acceleration being sensitive to inhibition by furosemide. However, Na_i^+ did not accelerate Rb^+ uptake in the NEM-treated cells, neither the total uptake nor its FS fraction, in contrast to the Rb^+ uptake seen in control cells (Fig. 13). Similarly, the FS K^+ loss induced by NEM (9 μmol · (ml · hr)$^{-1}$) was not affected by Na_i^+ (*data not shown*).

K^+ loss from NEM-treated cells was augmented by a factor of about 20. The enhanced K^+ loss was reduced by Rb_o^+ (Fig. 14, upper panel), in contrast to the K^+ loss in untreated cells which is accelerated or not affected by Rb_o^+ (Fig. 11). Rb^+ uptake and K^+ loss induced by NEM were partly blocked by furosemide, the inhibition increasing with Rb_o^+ (Fig. 14). The kinetic constants of Rb^+ uptake augmented by NEM and its FS component were calculated from the data shown in the lower panel of Fig. 14 using Eq. (1). The apparent $K_m Rb_o^+$, K_i and V_{max} values obtained are 23 and 31 mM Rb_o^+, $> 10^8$ mM and 1300 mM Rb_o^+, and 21 and 15 μmol Rb^+ uptake · (ml cells · hr)$^{-1}$, respectively. Accordingly, the apparent affinity for Rb_o^+ of the transport pathway chemically induced by NEM seems to be lower than that of the Na_o^+-independent component (*see* Table 3).

The pH dependence of Rb^+ uptake induced by NEM in choline media was studied at 50 mM Rb_o^+. In control cells, Na_o^+-independent FS Rb^+ uptake fell with rising pH to one-half (Table 5), in accordance with the data in Table 3. The Rb^+ uptake induced by NEM and its FS fraction, in contrast, showed the opposite change and increased more than twofold upon elevation of the pH from 6.6 to 8.0.

Ten μM bumetanide which blocks Na_o^+-independent Rb^+ uptake to a similar extent as 0.5 mM furosemide in normal human erythrocytes (Fig. 3) suppressed only one-half of the fraction of Rb^+ uptake induced by NEM which was furosemide sensitive (*data not shown*).

All these findings demonstrate that Rb^+ uptake chemically induced by NEM differs in a number of properties from the basal Na_o^+-independent FS Rb^+ uptake found in normal human erythrocytes.

Table 5. Effects of pretreatment with NEM on Rb^+ uptake of human erythrocytes in media containing 50 mM Rb^+ and 100 mM choline (chloride salts) as a function of the pH value (10 mM MOPS-Tris buffer, pH values measured at 37°C in the cell suspensions)[a]

pH	Pretreatment with NEM at pH 7.4	Control	Furosemide	Furosemide-sensitive
6.6	− (n = 7)	1.82 ± 0.27	1.10 ± 0.20	0.72 ± 0.09
	+ (n = 3)	10.2 ± 0.2	4.78 ± 0.28	5.43 ± 0.16
	NEM-augmented	8.67	3.68	4.71
7.4	− (n = 9)	1.55 ± 0.07	1.13 ± 0.11	0.43 ± 0.07
	+ (n = 3)	18.0 ± 0.9	6.61 ± 0.50	11.3 ± 1.4
	NEM-augmented	16.5	5.48	9.90
8.0	− (n = 5)	2.35 ± 0.28	1.98 ± 0.19	0.37 ± 0.06
	+ (n = 3)	20.7 ± 0.5	7.53 ± 0.05	12.6 ± 0.5
	NEM-augmented	18.4	5.55	12.2

[a] The cells were pretreated with 1.6 mM NEM in choline media at pH 7.4 at a molar ratio of NEM to hemoglobin monomer of 1.75. NEM was removed by 8 mM mercaptoethanol and washing prior to the uptake measurements. Mean values from the number of experiments indicated in parentheses (± 1 SD, donor 1). Uptake rates in μmol (ml cells · hr)$^{-1}$. The uptake period was 30 min.

Discussion

Action of External Cations on Furosemide-Resistant Rb^+ Uptake

One difficulty in interpreting the data obtained in the present experiments arose from the consistent observation that replacement of choline by Na^+ in the medium reduced furosemide-resistant (FR) Rb^+ uptake (*see* Tables 1 and 2 and Fig. 4). This effect was most pronounced at pH 8.0 (Fig. 6). In addition, FR Rb^+ uptake in choline and Na^+ media was not a linear function of Rb_o^+ but showed a small saturating component (Figs. 4 and 5). Similar observations have been made earlier [8, 44].

A first possible explanation of these phenomena was that Na_o^+ (and Rb_o^+) increased the inhibitory potency of furosemide. However, raising the drug concentration above the value of 0.5 mM routinely employed did not enhance the inhibitory effect. The same was seen with bumetanide (Fig. 3).

A second possibility that external cations such as Na^+ reduce the Rb^+ leakage rate constant relative to choline was investigated in Table 2. Indeed, all cations tested lowered FR Rb^+ uptake relative to choline media. The efficacy increased in the order $K^+ < Na^+ < Li^+ < Mg^{2+}$, in accordance with earlier observations [8, 44]. The sequence $Na^+ < Li^+$ is the reverse of that of the affinities of the cations for the Na^+ site of the Na^+-K^+ cotransport system [4] and Mg^{2+} is unlikely to interact with this site in a manner similar to Na^+. *Accordingly, the reduction of FR Rb^+ uptake induced by Na_o^+ and the other cations most probably results from a lowering of the Rb^+ inward leak relative to choline* (operationally defined as Rb^+ uptake in the presence of ouabain plus furosemide). A similar conclusion of Na_o^+ inhibiting a 'non-co-transport' pathway for K^+ (Rb^+) has been drawn by Chipperfield [8]. Likewise, the small saturating component of FR Rb^+ uptake (Figs. 4 and 5) can be attributed to a reduction of the Rb^+ leakage rate constant by Rb_o^+.

The alternative interpretation of the cations stimulating FR Rb^+ uptake relative to Mg^{2+} media in the order $Li^+ < Na^+ < K^+ <$ choline$^+$ cannot be excluded (for the action of other replacing cations *see* ref. [8]).

Components of FS Rb^+ Uptake at Normal Red Cell Volume

The results presented demonstrate that FS inward transport of Rb^+ in human erythrocytes exhibits two components in Na^+ media: one that is independent of Na_o^+ (Na_o^+-*independent*) and also seen in choline, K^+, Mg^{2+} or pure Rb^+ media, and a second that requires the presence of Na_o^+ (*Na_o^+ dependent*). This conclusion is in accordance with the classic observations of Wiley and Cooper [44] as well as with more recent reports on Na_o^+-independent Rb^+ or K^+ movements in which inhibition by either loop diuretics or by replacement of chloride with nitrate was applied to assess the transport system [8, 18, 32, 43].

The properties of the two components of FS Rb^+ uptake are compared in Table 6. The physiological function of a 1 : 1 K_o^+/K_i^+ exchange is not obvious. Attempts to demonstrate that H^+ may

Table 6. Comparison of properties of basal Na_o^+-independent and Na_o^+-dependent FS K^+ (Rb^+) uptake in human erythrocytes at normal cell volume

	Na_o^+-independent	Na_o^+-dependent	
Chloride-dependence	Yes	Yes	
Inhibition by furosemide (bumetanide), apparent K_i	6×10^{-6} (2×10^{-7} M)	1×10^{-6} (5×10^{-8} M)	Fig. 3
Contribution to total FS K^+ (Rb^+) uptake in 140 mM Na^+ – 5 mM Rb^+ media (at normal red cell Na^+ content)	25%	75%	Figs. 1 and 2
Contribution to total FS K^+ (Rb^+) uptake in Na^+ media	Increases with Rb_o^+, Na_i^+ and pH	Falls with Rb_o^+, Na_i^+ and pH	Tables 1 and 3 Figs. 4–6
Proposed mode of operation	1 : 1 K_o^+/K_i^+ exchange	1 : 1 Na^+-K^+ cotransport	
Apparent V_{max}	Falls with rising pH	Increases with rising pH	Table 3
Affinity for Rb_o^+	Low, apparent K_m increases with rising pH	High, apparent K_m independent of the pH	Table 3
Substrate inhibition by Rb_o^+	No	Yes	Table 3, Fig. 7
Na_i^+	Required but not extruded; apparent K_m 2–3 mM	Not required, inhibitory at high concentrations	Figs. 8 and 9
K_i^+	Required apparent K_m 20–30 mM	Not required	Fig. 9
Rb_o^+	Accelerates K^+ outward transport	Inhibits Na^+ and K^+ outward transport	Figs. 10 and 11
Na_o^+	Not required	Required can be replaced by Li_o^+	Tables 1 and 2 Figs. 4–9

serve as a substrate replacing K_o^+ have failed (*data not shown*).

Are the Two Components of FS Rb^+ Uptake Mediated by Two Modes of Operation of One Transport System?

A central question is whether the two components are mediated by two separate transport systems or represent two modes of operation of one and the same system. For duck erythrocytes which show two similar components, McManus preferred the second possibility [39, 40]. He presented a kinetic explanation of the paradoxical dependence on Na_i^+ of a Na_o^+-independent K_o^+/K_i^+ exchange mediated by a (1 Na^+ + 1 K^+ + 2 Cl^-) cotransport system. He proposed that the K_o^+/K_i^+ exchange represents a partial reaction of a transport system exhibiting an ordered binding on the outside (Na^+-Cl^--K^+-Cl^-) and the same order of debinding at the inside (glide symmetry [42]): Na^+ first on outside (E_1) and first off inside (E_2) (Fig. 15).

The partial reactions of the K_o^+/K_i^+ exchange in this model are reactions 3 to 8 forward and backward, reaction 6 explaining the absolute requirement of Na_i^+ and reactions 4 and 7 the need of Cl_o^- and Cl_i^- [41, 42]. The entire sequence of reactions 1 to 10 forward represents inward Na^+-K^+ cotransport.

The scheme would be consistent with *i*) the occurrence of inward Na^+-K^+ cotransport on top of K_o^+/K_i^+ exchange (or vice versa, *see* Fig. 9) (Na_o^+ promoting formation of E_1-Na-Cl from E_1, and Na_i^+ furthering the formation of E_2-Na-Cl-K-Cl from E_2-Cl-K-Cl) and *ii*) with the inhibition of outward Na^+-K^+ cotransport by Na_o^+ and K_o^+ (both ions driving the reaction sequence in the forward direction) (ref. [3] and Fig. 10). It predicts that the Na^+-K^+ cotransport and K_o^+/K_i^+ exchange mode of operation should vary strictly in parallel to each other both among erythrocytes of different donors with varying activities of the transport system (Fig. 1) and in fractions of erythrocytes of an individual donor separated according to density in which the old cells exhibit 1.6 to 5 times higher activity of the FS transport system than young cells (Fig. 2). A pH-dependent interconversion of the two transport modes could be explained by an inhibitory effect of H^+ on reactions 9, 10, 1 or 2 and an acceleration by H^+ of the reaction sequence 3 to 8 (with an apparent pK of close to 7.4 according to the data in Table 3). The suppression of Na_o^+-dependent FS Rb^+ uptake at high Rb_o^+ (Fig. 7) could be explained by Rb_o^+ driving reaction 3 to the right, thereby reducing the formation of E_1 from E_1-Na-Cl and, hence, the availability of E_1 and the rate of inward Na^+-K^+ cotransport (reactions 1 to 10). An alternative explanation comes from the kinetic analysis of the data assuming substrate inhibition to be responsible for the fall

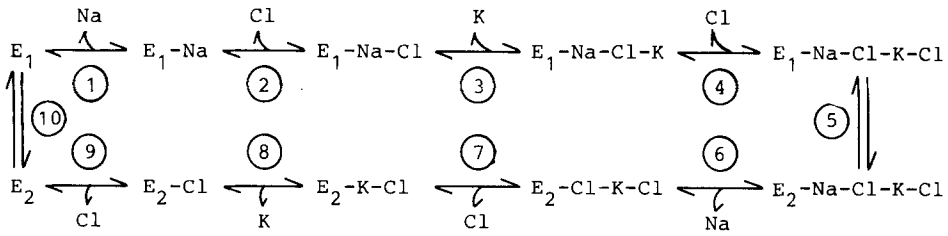

Fig. 15. Reaction scheme of an ordered binding and debinding (glide symmetry [42] in 1 Na^+ + 1 K^+ + 2 Cl^- cotransport as proposed by McManus [39, 40]. Partial reactions 3 to 8 promote a 1 : 1 K^+/K^+ exchange. E_1 = outside, E_2 = inside. For further details *see text*

of Na_o^+-dependent Rb^+ uptake at high Rb^+ concentrations. This phenomenon was most pronounced at pH 6.7 (Fig. 7 and Table 3). According to Cleland [10], substrate inhibition at high concentrations of one substrate ($>10 \times K_m$) in a two-substrate reaction results in most cases from combination of the inhibiting substrate with an enzyme form that it is not supposed to react with. If such a phenomenon were valid for the reaction scheme of Na^+-K^+ cotransport of Fig. 15 then the substrate inhibition of Na_o^+-dependent FS Rb^+ uptake (= Na^+-K^+ cotransport) seen at high Rb_o^+ would result from a combination of Rb_o^+ with the E_1 form of the enzyme, the E_1-Rb thus formed not being capable of traversing steps 2 to 8 of the reaction sequence.

The scheme would also allow for a FS 1 : 1 Na_o^+/Na_i^+ exchange mediated through the reversal of the partial reactions 1 to 6 in Fig. 15. Such an exchange, requiring Cl_o^- (reactions 2 and 4) and K_o^+ (reaction 3) but no K_i^+ and Cl_i^-, has been observed in duck erythrocytes [39, 40]. Canessa et al. [5] noticed a FS Na_o^+/Na_i^+ exchange in human erythrocytes in media free of K^+. This K_o^+-independent Na_o^+/Na_i^+ exchange, however, was stimulated by substitution of Cl^- by NO_3^- and, therefore, differs from the Cl_o^-- and K_o^+-dependent FS NA_o^+/Na_i^+ exchange seen in duck erythrocytes.

For the scheme in Fig. 15 to work the only transported forms are either the fully loaded or the completely empty carrier (reactions 5 and 10). This is more attractive than the scheme proposed by Canessa et al. [5] where a lot of slippage can occur. Furthermore, the mobility of the fully loaded carrier seems to be greater than that of the empty one since otherwise a reversal of the partial reactions 3–8 would not be seen.

However, there are two observations on human erythrocytes which are not easy to reconcile with the model of Fig. 15. Why does the apparent affinity for K_o^+ of the proposed reaction 3 increase in the presence of Na_o^+ (Table 3, ref. [8, 36])? The only reaction that Na_o^+ could promote in the scheme of Fig. 15 is to drive reaction 1 to the right, thereby providing more E_1-Na-Cl and increasing the maximum rate of Rb^+ uptake but leaving the affinity for Rb_o^+ combining with E_1-Na-Cl unaltered. To explain the observed increase in affinity for Rb_o^+ by Na_o^+ one has to postulate an external modifier site on the cotransport molecule distinct from the Na^+ transport site. The second question is: Why are the loop diuretics more effective in the presence of Na_o^+? One possible answer would be that the diuretics preferentially bind to E_1-Na or E_1-Na-Cl. However, Na_o^+ and K_o^+ mutually increase the inhibitory potency of furosemide and bumetanide, indicating that the E_1-Na-Cl-K form is the most stable inhibited conformation [32]. If the latter were the case, then Rb^+ uptake should be suppressed at a given concentration of the drugs to the same extent in the absence and presence of Na_o^+ in the scheme of Fig. 15. This, however, was not observed (Fig. 3). Again, an external modifier site for Na^+ has to be postulated to explain the increased inhibitor potencies induced by Na_o^+.

A modifier site for Na_o^+ is not required in a scheme in which the transport molecule is thought to exhibit a pH-dependent allosteric equilibrium between two conformations, conformation R promoting a strictly coupled, bidirectional Na^+ + K^+ + $2Cl^-$ cotransport, and conformation T a chloride-dependent 1 : 1 K_o^+/K_i^+ exchange requiring Na_i^+ (which is not transported to or released at the outside). The allosteric equilibrium is thought to be controlled by a titratable group with a pK near pH 7.4. The T form has to have a lower affinity for K_o^+ and loop diuretics than the R conformation. Such a scheme would be in accordance with all of the properties of FS Na^+ and K^+ transport discussed above, with the exception of a K_o^+-dependent FS NA_o^+/Na_i^+ exchange which remains to be demonstrated in human erythrocytes.

Regardless of whether anyone of the two schemes discussed above accurately described the Cl^--dependent, FS Na^+-K^+ transporter of human erythrocytes, the data presented in Results are consistent with the view that Na_o^+-dependent FS Rb^+

uptake and FS Rb^+ uptake through Na_o^+-independent Rb_o^+/K_i^+ exchange are mediated by *two modes of operation of one and the same system* in normal human erythrocytes at normal cell volume. If the two components of FS Rb^+ uptake were mediated by two separate systems one would expect that the activities of the two would vary independently of each other in erythrocytes with different activities of the systems. The strict parallelism between the two components reported in Figs. 1 and 2 would thus lead to the postulate of a coupling mechanism linking the functionally expressed activities of two separate systems tightly together.

The parallel changes of Na^+-K^+ cotransport and of K_o^+/K_i^+ exchange seen in red cells with individually different activities of the FS transport system seem to preclude the possibility that the disparity of results obtained in studies on red cell Na^+-K^+ cotransport activity in essential hypertensive patients is caused by the use of either efflux or influx techniques, assessing different modes of operation of the transport system.

FS Rb^+ Uptake in NEM-Treated and Swollen Erythrocytes

Dunham et al. [18, 43] as well as Kaji and Kahn [32] favored the view of Na_o^+-dependent and Na_o^+-independent FS (chloride-dependent) Rb^+ uptake being mediated by two separate pathways. They concluded that NEM activates the pre-existing Na_o^+-independent system. This idea is based on the observation that pretreatment of human erythrocytes with the sulfhydryl reagent N-ethylmaleimide (NEM) induces a Cl^--dependent, Na_o^+-independent Rb^+ uptake. The pathway accelerated by NEM is inhibited in part by furosemide and exhibits a low affinity for Rb_o^+ [11, 36–38]. Lauf et al., in contrast, suggested that NEM induces a new or latent Cl^--dependent K^+ transport pathway which is normally silent [36, 37]. This proposal was based on the findings that K^+ fluxes of cells treated with NEM *i*) were only partly inhibited by furosemide and bumetanide, *ii*) were sensitive to cell volume changes whereas the basal Cl^--dependent Rb^+ transport was not, and *iii*) were not correlated in magnitude to the interindividually different basal transport rates [36, 38].

According to the data of Fig. 12 and recent results of Kaji and Amblard [31] the second argument is questionable because Na_o^+-independent FS Rb^+ uptake in human erythrocytes proved to be volume sensitive, as it is in sheep erythrocytes [33–35]. The volume-sensitive pathway, however, seems to differ from the basal Na_o^+-independent pathway functionally expressed at normal cell volume inasmuch as it is independent of Na_i^+ and can promote net movements of K^+ plus Cl^- (ref. [31] and *unpublished results* obtained with rat erythrocytes).

The present data provide further evidence that Rb^+ uptake induced by NEM and its FS fraction differ in properties from the basal Na_o^+-independent component found in human erythrocytes at normal cell volume: *i*) the NEM-induced uptake is independent of Na_i^+ between 0.1 and 20 μmol · ml^{-1} (Fig. 13) whereas the presence of Na_i^+ is a prerequisite for the basal transport to occur, *ii*) in media containing 50 mM Rb^+ and 100 mM choline, the Rb^+ uptake induced by NEM rose more than twofold upon increasing the pH from 6.7 to 8.0, whereas the basal uptake fell to about one-half upon the same pH change (Table 5), thus suggesting an opposite pH dependency of the two phenomena, *iii*) Rb_o^+ inhibits K^+ release induced by NEM (Fig. 14), thus excluding an induction of Rb_o^+/K_i^+ exchange by NEM. Similar results have been obtained using sheep erythrocytes [33]. *iv*) The inhibitory potency of bumetanide on the basal Na_o^+-independent Rb^+ uptake in untreated human erythrocytes was found to be greater (apparent K_i 1.9×10^{-7} M, Fig. 3) than that reported for the Rb^+ uptake induced by NEM (apparent K_i 2×10^{-4} M, ref. [36]). *v*) Finally, reduction of cell Ca^{2+} by treatment with 10 μM A23187 and 1 mM EGTA in media containing 0.2 mM $MgCl_2$ did accelerate the basal Na_o^+-independent FS Rb^+ uptake in human erythrocytes at best by about 15% (B. Engelmann and J. Duhm, *unpublished results*), in contrast to the 10-fold increase of Cl^--dependent Rb^+ uptake caused by Ca^+ depletion in low K^+ sheep red cells [35]. The latter finding indicates that the basal Na_o^+-independent Rb^+ uptake in human and low K^+ sheep red cells exhibit different properties.

Such a multitude of disparities in properties strongly suggest that the Na_o^+-independent, chloride-dependent K^+ transport pathways present in human erythrocytes under basal conditions and after treatment with NEM are not mediated by the same transport system. It is concluded, therefore, that the K^+ transport pathway chemically induced by NEM does not result from an activation of the Na_o^+-independent basal component but is rather due to an induction of a latent Cl^--dependent K^+ transport pathway normally nonexisting in human erythrocytes. Possibly, this pathway can also be activated by oxidation of membrane sulfhydryl groups [20].

Conclusions

According to several reports [3–5, 8, 12, 23, 30–32, 36–38, 43, 44] and the present data, three modes of

FS, Cl^--dependent K^+ transport seem to exist in *human* erythrocytes: *i*) bidirectional (1 Na^+ + 1 K^+) cotransport, *ii*) 1 : 1 K_o^+/K_i^+ exchange, independent of Na_o^+ but requiring Na_i^+, and *iii*) bidirectional K^+ transport independent of both Na_o^+ and Na_i^+ in swollen cells. The present experiments provide indirect evidence that modes *i*) and *ii*) are mediated by the same transport system. The relation of mode *iii*) to the other two has not yet been studied in detail. The pathway chemically induced by NEM is most probably different from modes *i*) and *ii*). Its possible connection with the swelling-induced K^+ transport pathway of human erythrocytes remains to be elucidated.

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