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$Na^+/K^+/Cl^-$ cotransport in resealed ghosts from erythrocytes of the Milan hypertensive rats

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The erythrocytes (RBC) of the Milan hypertensive rats (MHS) have a smaller volume and faster $Na^+/K^+/Cl^-$ cotransport than RBC from normotensive controls (MNS). The difference in $Na^+/K^+/Cl^-$ cotransport is no longer present in inside-out Vesicles (IOV) of RBC membrane. To differentiate between cytoplasmic or membrane skeleton abnormalities as possible causes of these differences, Resealed ghosts (RG) were used to measure ion transport systems. The following results have been obtained: (1) RG from MHS have a smaller volume than MNS (mean ± S.E. 20.7 ± 0.45 vs. 22.09 ± 0.42 fl, $P < 0.05$). (2) RG showed a bumetanide-sensitive Na efflux that retains the characteristics of the $Na^+/K^+/Cl^-$ cotransport of the original RBC: it is K^+- and Cl^--sensitive and dependent on the intracellular Na^+ concentration. (3) The $Na^+/K^+/Cl^-$ cotransport was faster in RG from MHS than in those from MNS (mean ± S.E. 0.095 ± 0.01 vs. 0.066 ± 0.01 rate constant h^{-1}, $P < 0.01$). These results, together with those of IOV, support the hypothesis that an abnormality in the membrane skeletal proteins may play a role in the different $Na^+/K^+/Cl^-$ cotransport modulation between MHS and MNS erythrocytes.

Introduction

Among the many abnormalities of cell ion-transport systems so far described in essential hypertension, those of the erythrocyte $Na^+/K^+/Cl^-$ cotransport are particularly interesting for the following reasons: (1) they have been found both in humans with essential hypertension [1–5] and in rat strains with genetic forms of hypertension [6–9]. (2) One of these strains, the Milan hypertensive rat strain (MHS), showed a faster erythrocyte $Na^+/K^+/Cl^-$ cotransport before the development of hypertension than the normotensive controls MNS [10], and this characteristic was demonstrated to be primarily determined within the stem cell [11] and genetically associated with the blood pressure levels on F2 hybrids derived from F1 (MHS × MNS) rats [11]. (3) The $Na^+/K^+/Cl^-$ cotransport alterations of MHS erythrocytes are similar to those found in luminal membrane of the thick ascending limb cells [12] or in vascular smooth muscle cells [13], namely the rate of the $Na^+/K^+/Cl^-$ cotransport measured in the MHS cells is faster than in the corresponding cells from the control normotensive rats (MNS). The similarities between erythrocytes and kidney cells can be relevant to the understanding of the pathogenetic mechanisms of MHS hypertension, since high blood pressure can be transplanted between MHS and MNS with the kidney [14,15] and the pressor effect of MHS kidney seems to be due to an accelerated Na^+ transport across the tubular epithelium [16].

All these observations suggested that the faster $Na^+/K^+/Cl^-$ cotransport of MHS erythrocytes could be due to the same cell alteration that, at kidney level, is involved in the development of hypertension. Therefore, the understanding of the cellular mechanisms responsible for the altered $Na^+/K^+/Cl^-$ cotransport of MHS erythrocytes should also be useful for detecting the genetic renal defect causing hypertension in MHS rats and, at least, in those hypertensive patients with faster erythrocyte $Na^+/K^+/Cl^-$ cotransport and increased tubular Na^+ reabsorption [2,17].

In previous studies we have shown that the faster $Na^+/K^+/Cl^-$ cotransport of MHS erythrocytes is due to a higher affinity for internal Na^+ [18]. These differ-

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ences between MHS and MNS disappear in erythrocyte inside-out vesicles (IOV), where cytoplasmic and membrane skeletal proteins are removed [18]. This suggests that the altered $Na^+/K^+/Cl^-$ cotransport is not due to an abnormality of the transmembrane protein(s) but to some regulatory intracellular factor(s) or skeletal membrane protein(s) which affect the $Na^+/K^+/Cl^-$ cotransport activity of intact MHS erythrocytes.

The aim of the present study was to discriminate between these two possibilities. We approached this problem using erythrocyte resealed ghosts (RG) from both strains, since this cellular preparation maintains an intact skeletal membrane protein network and allows the substitution of the cellular cytosol with artificial milieu [19–22].

The results we obtained are: (a) rat RG show a bumetanide, K^+ and Cl^--sensitive Na^+ efflux with the same characteristics of the $Na^+/K^+/Cl^-$ cotransport of fresh erythrocytes; (b) at physiological and comparable intracellular Na^+ concentrations, $Na^+/K^+/Cl^-$ cotransport activity is higher in MHS than in MNS RG; (c) as in intact erythrocytes [10], MHS RG have a lower volume than MNS. Therefore, together with the results obtained from IOV [18], these results exclude the possibility that some cytosolic factor can alter the MHS $Na^+/K^+/Cl^-$ cotransport and favor the hypothesis that the $Na^+/K^+/Cl^-$ cotransport is differently modulated by the membrane skeleton in MHS and MNS rats.

Materials and Methods

Animals. The experiments were performed on RBC obtained from male hypertensive rats (MHS/Gib) and their normotensive controls (MNS/Gib), derived from our original breeding colony (Prassis, Research Institute Sigma-Tau, Settimo M., Milan, Italy). Animals were used at 7–8 weeks of age. Systolic blood pressure, measured by tail cuff method, averaged 168.4 ± 1.1 mmHg in MHS and 132.1 ± 1.2 mmHg in MNS. Both strains had free access to food and water and were maintained on normal sodium diet (0.5% NaCl (w/w), Altromin MT Rieper, Vandois, Italy), up to the moment of the experiments.

Medium composition in mM. Phosphate-buffered saline (PBS): NaCl 140; Phosphate buffer 10 (pH 7.4) at room temperature. Hypotonic lysis solution: $MgSO_4$ 4; ATP(Mg) 4; dithiothreitol 1; diisopropyl fluorophosphate 0.4; glutathione (reduced form) 1 (pH 6.0) at 0°C. Reversal Na^+-free solution (10×): KCl 1045; glucose 20; 3-(*N*-morpholino)propanesulfonic acid (Mops)-tris(hydroxymethyl)methylamine (Tris) buffer 100 (pH 7.3) at 37°C. Sodium loading solution: NaCl 35; KCl 80; glucose 5; ouabain 10; Mops-Tris buffer 10 (pH 7.4) at 37°C. Sucrose cushion: sucrose 45% (w/v) into sodium loading solution. Choline/sucrose medium: choline chloride 80; sucrose 75; $MgCl_2$ 1; glucose 10; Mops-Tris buffer 10 (pH 7.4) at 37°C

When required by the experimental protocol, chloride was substituted by either equimolar nitrate or sulphate ions, and potassium (or sodium) were substituted by choline ions. Choline nitrate and choline sulfate were prepared neutralizing the base (50% in water) with concentrated HNO_3 or H_2SO_4 [23]. Reagents were purchased from Sigma, Aldrich and Merck.

Preparation of resealed ghosts. From each rat, 10 ml of blood was collected in a heparinized syringe, sedimented at 700 × *g* for 15 min at 4°C and the plasma and buffy coat removed. RBC were never pooled and each measurement referred to a single animal.

RG were prepared following the method of Schwoch and Passow [22], with some modifications. Briefly, the RBC were washed three times in PBS. One ml of the final packed RBC was lysed in 10 volumes of the hypotonic lysis solution, kept on ice for 5 min to complete the hemolysis, then sedimented at 32000 × *g* for 10 min at 4°C. The supernatant was removed and new hypotonic lysis solution was added. This allows a minimum of 70-fold dilution of the soluble cytosolic component. 1 ml of the concentrated Reversal Na^+-free solution was added. The pH of the final isosmotic medium was adjusted to 7.3 during 10 min incubation at 0°C. The tubes were then warmed to 37°C for 45 min. At the end of the resealing period the ghosts were sedimented and the supernatant replaced with sodium loading solution for some minutes, according to the experimental protocol. For most of the experiments, we incubated for 5 min at 37°C, which allows the entrance of Na^+ up to a physiological concentration (5–7 mmol Na^+/l RG) (Table 1). For the experiments performed to determine the activation of Na^+ efflux by intracellular Na^+, aliquots of RG were incubated in the sodium loading solution for increasing times, in order to achieve the desired intracellular Na^+ concentrations.

The ghosts were pelleted, layered on the top of a sucrose cushion and sedimented for 1 h at 28000 × *g*. The 'resealed' ghosts were collected at the interface, while the 'leaky' ghosts were pelleted. Aliquots of 'resealed' ghosts were lysed in bidistilled water for hemoglobin measurements.

Na^+ efflux measurement. The studies were performed by the 'zero-trans' method, as already described for intact erythrocytes [10]. The RG were washed three times in the choline/sucrose solution. Washed RG were incubated in choline/sucrose medium with the following additions: tube 1: KCl 5 mM; tube 2: ouabain 5 mM and tube 3: ouabain 5 mM + bumetanide 0.5 mM (KCl was omitted in tube 2 and 3 to favor the ouabain binding to rat Na^+/K^+-

ATPase). The bumetanide concentration was chosen according to the dose-response curve in RG (see results and Fig. 3) as the lowest dose giving the maximum inhibition of Na^+ efflux. The final hematocrit was 1–2%. Incubation was carried out at 37°C in a shaking water bath. Aliquots were taken in triplicate after 0, 5 and 15 min and 0, 10 and 20 min respectively for the total Na^+ efflux and the ouabain, bumetanide-sensitive Na^+ effluxes and immediately spun for 30 s in an Eppendorf Minifuge at 12000 rpm. The supernatant was removed for Na^+ determination by atomic absorption (Perkin Elmer 1100B). Aliquots of RG suspension were lysed in bidistilled water for measurement of the intracellular Na^+ and K^+ concentrations by atomic absorption spectrophotometry and for hemoglobin content. Na^+ efflux was calculated by the least-squares method from the points obtained at the different sampling times. The Na^+/K^+-pump activity was calculated as the ouabain-sensitive Na^+ efflux; the $Na^+/K^+/Cl^-$ cotransport activity as the ouabain-resistant, bumetanide-sensitive Na^+ efflux and the Na^+ passive permeability as the residual Na^+ efflux in presence of ouabain and bumetanide. The intra-assay coefficient of variability was 8.6% for pump measurements, 10.5% for cotransport and 2.4% for passive permeability.

K^+ efflux measurements. RG were prepared as described above, except for the presence of Na^+ ions replaced by choline in each solutions. Washed RG were incubated in the same choline/sucrose solution, as for Na^+ efflux measurements, for 0, 10 and 20 min at 37°C, in the presence of 5 mM ouabain and with or without 0.5 mM bumetanide. Aliquots were taken in triplicate and the supernatant was separated by Eppendorf centrifugation and measured for K^+ concentration by atomic absorption spectrophotometry.

Cell volume measurement. Erythrocyte and RG volumes were measured in a Coulter Counter ZM connected to a Multichannel Analyzer 256 (Coulter Electronics, UK) as previously described [10].

Membrane proteins. The membrane protein composition of RG of MHS and MNS rats was analyzed by SDS-PAGE, as described previously [24].

Hemoglobin content. The hemoglobin content was evaluated measuring absorbance of lysate at 540 nm in a Jasco 77850 spectrophotometer.

Acetylcholinesterase assay. The assay was performed as described by Steck and Kant [20].

ATP measurements. ATP content was measured with the Sigma ATP kit.

Statistics. All transport values were expressed as

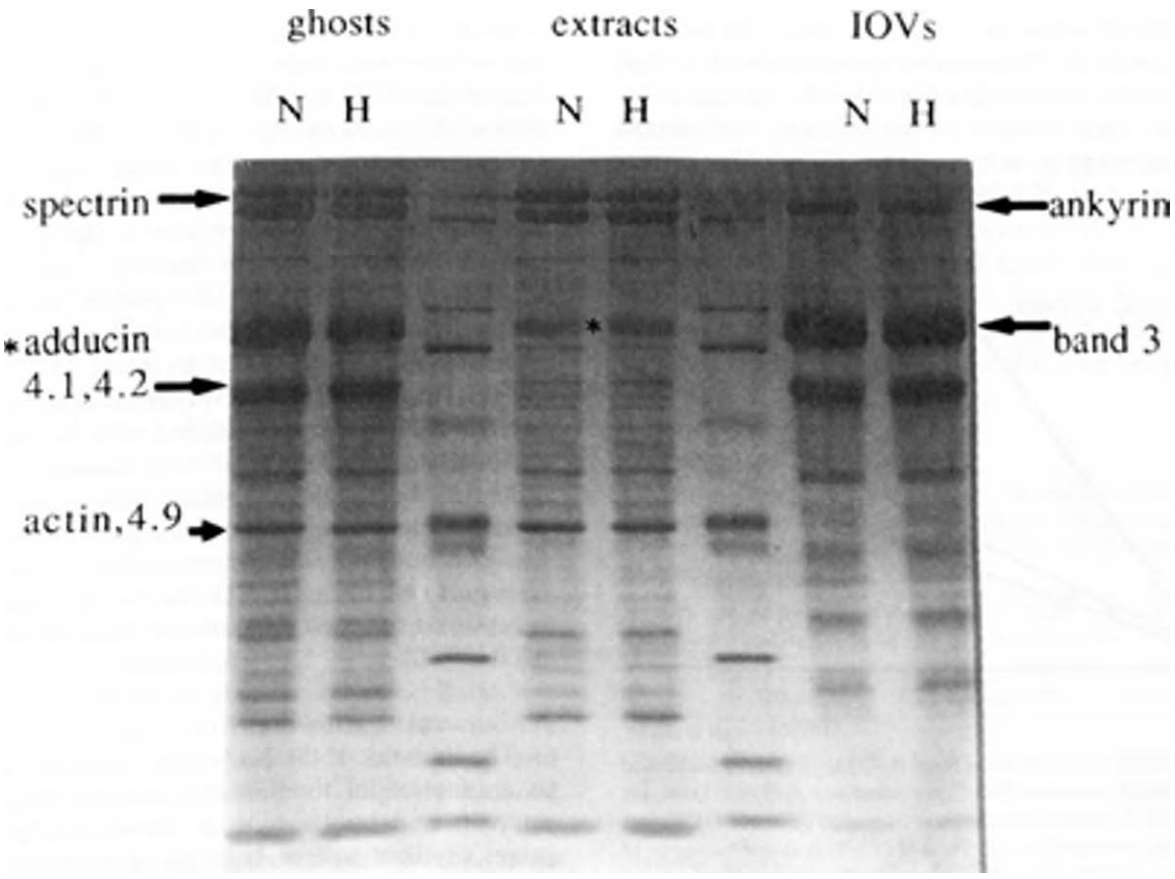

Fig. 1. SDS-PAGE of membranes from MNS (N) and MHS (H) erythrocytes. Ghosts, 40 μg protein; extracts, low-ionic-strength extracts from ghost preparation, 20 μg proteins. (*) indicates adducin. IOV, Inside-out vesicles, 20 μg proteins. Samples are intercalated by molecular weight standards. The gel is stained with Coomassie blue R250.

rate constant (h^{-1}), to normalize individual variability in intracellular Na^+ content and cell volume and allow comparison between RBC and RG values. IC_{50} for the bumetanide dose-response curves and kinetic parameters for activation curves were calculated by a non-linear fitting computer program (SIGMAPLOT, Jandel Scientific, USA). Data are expressed as the mean ± S.E. Statistical evaluation was performed by Student's *t*-test.

Results

Rat RG membrane protein pattern, volume and intracellular ion concentrations

As shown in Fig. 1, the electrophoretic pattern of RG membranes of both strains was similar and no signs of proteolitic degradation of the main integral and membrane skeletal proteins were detected. The residual hemoglobin content was on average 15% of the basal content in RG of both strains. They appeared to be sealed to macromolecules as no further release of hemoglobin [21] or of entrapped radiolabelled IgG were detected after several washes (data not shown). The ghosts were defined as 'sealed' to transportable ions and, therefore, used for flux measurements, when the internal K^+ content, measured after three washes in choline/sucrose (Na^+ and K^+ free) medium at 4°C and just before the efflux measurement was comparable with the K^+ concentration present in the reversal solution (Table I). Potassium ions were chosen, since RG are usually characterized by high K^+ permeability [25–27] and an activation of the K^+/Cl^- cotransport has been reported as consequence of stretching during ghost preparation [25,26,28]. The separation of the

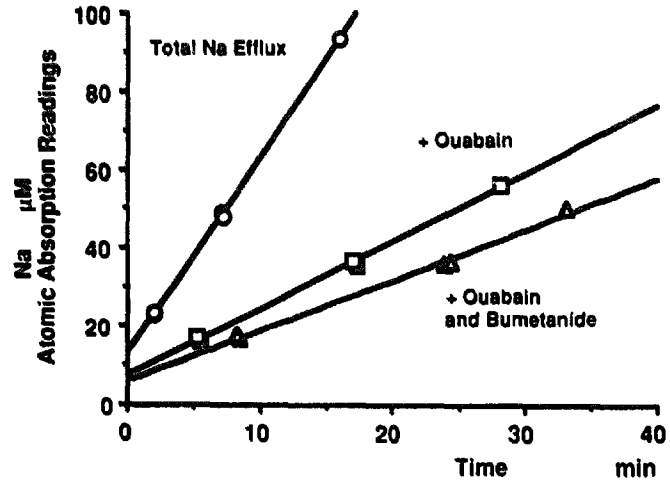

Fig. 2. Time-course of Na^+ efflux from RG. The RG were incubated in choline/sucrose medium (Na^+-free medium) at 37°C. Data are taken from 1 of 15 similar experiments averaged in Table III. Points were measured in duplicate. (○) Total Na^+ efflux in the presence of 5 mM KCl, $r = 0.999$. (□) Ouabain-resistant Na^+ efflux in the presence of 5 mM ouabain, $r = 0.999$. (△) Ouabain- and bumetanide-resistant Na^+ efflux in presence of 5 mM ouabain and 0.5 mM bumetanide, $r = 0.991$.

TABLE I

Volume and intracellular composition of washed intact RBC and RG from MHS and MNS (concentration: mmol / l cell)

Results are expressed as means + S.E. (RBC, $n = 15$; RG, $n = 15$). Statistical significance by means of Student's *t*-test.

	Volume (fl)	$[Na]_{in}$	$[K]_{in}$	$[ATP]_{in}$
Intact RBC				
MHS	41.01 ± 0.46	3.12 ± 0.13	110.6 ± 5.5	1.78 ± 0.07
	$P < 0.01$	$P < 0.05$	N.S.	N.S.
MNS	42.95 ± 0.41	3.49 ± 0.12	112.2 ± 3.6	1.72 ± 0.08
Released ghosts				
MHS	20.7 ± 0.45	6.22 ± 0.099	100.78 ± 2.16	0.631 ± 0.058
	$P < 0.05$	N.S.	N.S.	N.S.
MNS	22.09 ± 0.42	6.042 ± 0.149	101.53 ± 1.75	0.706 ± 0.040

sealed from the leaky ghosts improved the preparation, since the K^+ content in the total ghost population, before the sucrose cushion, ranged from 15 to 30 mmol/l RG, while in the resealed ghosts harvested after the sucrose cushion ranged from 80 to 110 mmol/l RG. The presence of sealed inside-out membrane was checked in some preparations of both strains by the acetylcholinesterase assay, giving almost 100% of re-sealed right-side out ghosts.

The characteristics of RG and erythrocyte from MHS and MNS rats are reported in Table I. The mean RG volume was reduced in both strains compared to that of the RBC, but MHS RG volume was lower than that of MNS, as in intact erythrocytes. After resealing and before starting with Na^+ flux measurements, MHS and MNS RG showed similar intracellular Na^+ (Na_i^+), K^+ (K_i^+) and ATP concentrations. The K_i^+ concentration was comparable with that of intact erythrocytes, while Na_i^+ was higher, but still within the physiological range. The lack of difference in Na_i^+ between MHS and MNS RG, as compared to intact RBC, was probably due to the different experimental conditions. The MHS and MNS RG were loaded with the same medium and maintained at 4°C during washes, in order to minimize their leakyness, while RBC were handled at room temperature. ATP concentration was reduced in the RG, however this concentration can maintain ion transport systems, as in RG the Na^+/K^+-pump activity is reported to reach saturation at 0.5 mM intracellular ATP [29,30].

Na^+ transport systems in RG

The linearity of the Na^+ efflux from RG of MNS rat as a function of time in the absence or presence of ouabain and bumetanide is shown in Fig. 2. As in intact erythrocytes, at least three components of the total Na^+ efflux were detectable in RG: (1) a ouabain-sensitive one, dependent on external potassium which could be mediated by the Na^+/K^+ pump; (2) a

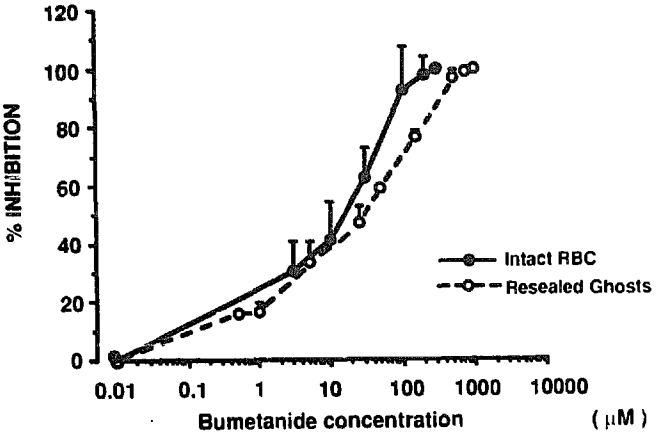

Fig. 3. Inhibitory activity of bumetanide on ouabain-resistant Na^+ efflux from normotensive rat RBC (●) and RG (○). The cells were incubated in choline/sucrose medium (Na^+-free medium) at 37°C in the presence of 5 mM ouabain. Points are mean ± S.E., $n = 4$.

ouabain-insensitive, bumetanide-sensitive one, which could be mediated by the $Na^+/K^+/Cl^-$ cotransport and (3) a ouabain and bumetanide-insensitive one, which could represent the Na^+ passive permeability. The presence of a ouabain-sensitive Na^+/K^+ ATPase activity (Na^+/K^+ pump) in erythrocyte ghosts has already been well described [29,31,32].

We have further investigated the ouabain-insensitive, bumetanide-sensitive component to verify if it satisfied all the requirements for Na^+ transport through a $Na^+/K^+/Cl^-$ cotransport [33,34]. Fig. 3 shows the percent of inhibition of Na^+ efflux as function of the extracellular bumetanide concentrations in both intact erythrocytes and RG from MNS rats. The two curves were not statistically different ($P > 0.1$ by SIGMAPLOT parameters) and maximum inhibition was achieved at 0.2 mM in RBC and 0.5 mM in RG. On the basis of these curves, we decided to work in presence of 0.5 mM bumetanide for all the measurements of the bumetanide-sensitive Na^+ efflux in rat RG. The IC_{50} was 14.1 ± 3.28 μM for intact RBC ($n = 4$) and 23.6 ± 8.02 μM for RG ($n = 5$) (not significantly different, $P > 0.15$). Similar results were obtained with MHS RBC (IC_{50} 16.6 ± 7.4 μM, $n = 4$) and RG (IC_{50} 25.3 ± 7.6 μM, $n = 4$).

We measured the dependency of the RG bumetanide-sensitive Na^+ efflux on the presence of extracellular and intracellular K^+ and Cl^-. As reported in Table II, in the absence of intracellular K^+, the bumetanide-sensitive Na^+ efflux in chloride medium was almost abolished (79% inhibition) and chloride substitution with nitrate or sulphate reduced the Na^+ efflux by 56.7 and 62.5%, respectively. No significant difference was detected in RG volume in presence or absence of Cl^-. Therefore, at least a significant portion of the bumetanide-sensitive Na^+ efflux is also Cl^-- and K^+-dependent and is consistent

TABLE II

Effect of ion substitution on the ouabain-resistant, bumetanide-sensitive Na^+ efflux in RG from MHS (rate constant h^{-1})

Results are expressed as means + S.E., $n = 4$.

Control (Na–K–Cl)	No potassium (Na^+/choline/Cl)	No chloride	
		(Na^+/K^+/nitrate)	(Na^+/K^+/sulfate)
0.104 ± 0.01	0.021 ± 0.01	0.045 ± 0.02	0.039 ± 0.06

with the original hypothesis that in rat RG Na^+ efflux mediated by the $Na^+/K^+/Cl^-$ cotransport is present.

Characteristics of bumetanide-sensitive Na^+ transport of MHS and MNS RG

Table III shows the Na^+ efflux through the ouabain-sensitive transport, the bumetanide-sensitive transport and the Na^+ passive permeability of intact RBC and RG prepared from MHS and MNS rats whose intracellular Na^+-, K^+- and ATP concentrations were reported in Table I. The bumetanide-sensitive Na^+-transport rates of both RBC and RG were comparable within strains but were significantly higher in MHS than in MNS, both in RBC and RG. The ouabain-sensitive transport rates were similar between the two strains both in RBC and in the RG. The residual Na^+ efflux (passive permeability) increased in RG as a consequence of the ghost preparation procedure, but the extent could still be considered comparable to that observed in the intact RBC [21]. Also, a statistically significant higher Na^+ passive permeability was found in MHS than in MNS RG.

We previously demonstrated that the faster $Na^+/K^+/Cl^-$ cotransport of fresh MHS erythrocytes was due to a higher affinity for internal Na, in comparison with MNS [18]. To verify if this kinetic characteristic was also maintained in RG, we measured the

TABLE III

Ouabain-sensitive (Na^+/K^+ pump), bumetanide-sensitive and ouabain-resistant ($Na-K-Cl$ cotransp), ouabain and bumetanide-resistant Na^+ efflux (pass. perm.) in intact RBC and RG from MHS and MNS rats (rate constant h^{-1})

Results are expressed as means + S.E. (RBC, $n = 15$; RG, $n = 15$). Statistical significance by means of Student's t-test.

	Pump	Cotransp	Pass. perm.
Intact RBC			
MHS	0.676 ± 0.02	0.119 ± 0.02	0.105 ± 0.01
	N.S.	$P < 0.02$	N.S.
MNS	0.659 ± 0.03	0.063 ± 0.01	0.107 ± 0.01
Resealed ghosts			
MHS	0.632 ± 0.07	0.095 ± 0.01	0.445 ± 0.02
	N.S.	$P < 0.01$	$P < 0.05$
MNS	0.650 ± 0.07	0.066 ± 0.01	0.388 ± 0.02

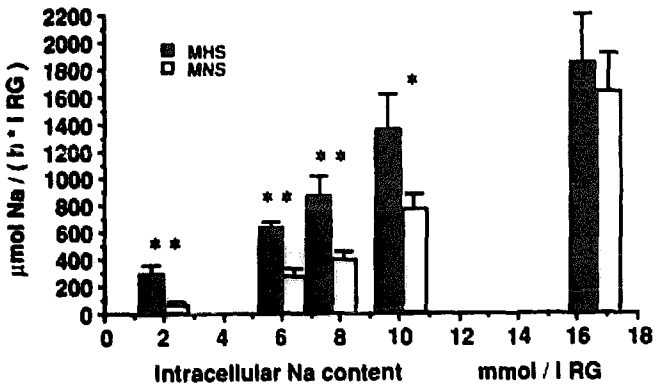

Fig. 4. Activation of bumetanide-sensitive Na^+ efflux ($Na^+/K^+/Cl^-$ cotransport) by intracellular Na^+ content in RG from MHS (■) and MNS (□) male rats. Data are means ± S.E., $n = 4$. * $P < 0.05$, ** $P < 0.01$ by means of Student's t-test.

bumetanide-sensitive Na^+ transport at increasing Na_i. Because of technical difficulties in obtaining more than 5 different Na^+ concentrations from the same rat RG preparation, the stimulation of bumetanide-sensitive efflux by Na_i^+ shown in Fig. 4 was obtained from the average of different partial activation curves from several rats. The two curves are sigmoidal in shape, as previously reported for the Na^+ activation curves of the $Na^+/K^+/Cl^-$ cotransport in rat and human RBC [3,4,9,18]. The bumetanide-sensitive Na^+ transport was significantly faster in MHS RG than in MNS in the 'physiological' range of Na_i^+ concentrations (2–10 mmol/l RBC) and became similar at higher Na^+ concentrations. Similar V_{max} (MHS 2319 ± 694 µmol Na^+/(h per litre RG); MNS 2817 ± 1206 µmol Na^+/(h per litre RG)) and lower K_m in MHS RG (9.14 ± 3.52 mmol/l RG) than in MNS RG (15.38 ± 7.14 mmol/l RG) can be calculated (SIGMAPLOT) from the average data reported in Fig. 4. The Hill coefficient n was 1.83 ± 0.59 in MHS RG and 1.87 ± 0.45 in MNS RG. These results are in accordance with the higher affinity for Na_i^+ of RBC $Na^+/K^+/Cl^-$ cotransport already demonstrated for MHS, compared to MNS (K_m MHS 10.3 ± 0.57 mmol/l RBC; MNS 19.2 ± 0.87 mmol/l RBC, $P < 0.001$, Hill coefficient $n = 2$ [18]).

TABLE IV

Cell volume and Na^+-independent, ouabain-resistant K^+ efflux in RG from MHS and MNS rats (rate constant h^{-1})

The RG were loaded with K^+ and choline in absence of Na^+ (see Methods). Bumetanide 0.5 mM. Results are expressed as means + S.E., $n = 5$. Statistical significance by means of Student's t-test.

	Resealed ghost volume	Bumetanide-sensitive	Bumetanide-resistant
MHS	20.47 ± 0.52	0.738 ± 0.04	0.869 ± 0.05
	$P < 0.05$	N.S.	N.S.
MNS	22.63 ± 0.54	0.662 ± 0.06	0.756 ± 0.07

K^+ permeability

We measured the K^+ efflux in MHS and MNS ghosts resealed in the absence of Na^+ (isosmolarity was maintained by adding choline instead of Na^+), in the presence of ouabain (5 mM), with or without bumetanide (0.5 mM). Table IV reports the Na^+-independent, ouabain-resistant, bumetanide-sensitive and ouabain and bumetanide-resistant K^+ efflux and the volume of MHS and MNS RG. RG volumes were lower in MHS than MNS and comparable with those reported in Table I, where intracellular Na^+ was present. The differences in both bumetanide-sensitive and bumetanide-resistant K^+ efflux in the two strains were not statistically significant.

Discussion

Three main findings emerge from this study: (1) the volume differences between MHS and MNS erythrocytes are maintained in RG; (2) RG from rat erythrocyte have a K^+- and Cl^--dependent Na^+ efflux, inhibited by bumetanide, similar to the Na^+ efflux through the $Na^+/K^+/Cl^-$ cotransport of intact RBC. Therefore, we will refer to this pathway as outward $Na^+/K^+/Cl^-$ cotransport; (3) the difference in RBC $Na^+/K^+/Cl^-$ cotransport rate between MHS and MNS rats is maintained also in RG, the MHS cotransport rate being higher than in MNS preparations.

Volume and ion composition of rat RG

The RG volume of both strains was 50% smaller as compared to intact RBC. This was expected on the basis of the volume ratio between RBC suspension and lysing medium (1 : 10, twice) and the delayed time from the lysis at 0° and the resealing at 37°C (15 min). Nash et al. [35] demonstrated that the final RG volume is strictly dependent on the ratio of lysate to resealing medium osmolality (the smaller the ratio, the smaller the volume) and on the delayed time from lysis to resealing (the longer the delay, the smaller the volume). Under conditions similar to those applied in our study the final RG volume was 50–60% smaller as compared to the original cell volume. We chose these conditions for lysis and resealing to minimize the influence of cytosolic factors.

MHS RG showed a statistically significant smaller volume than MNS, as for RBC, despite the reduction of the RG volume, as compared to the intact cells (Table I). This difference seemed due neither to different intracellular ion concentrations nor to a different energetic state, since these conditions were comparable in the two RG preparations (Table I). The different RG volume might be linked to the different Na^+ efflux through the $Na^+/K^+/Cl^-$ cotransport (Table III and Fig. 4), but this is not likely, since RG volume was different also when the ghosts were loaded only with

K^+, in the absence of Na^+ and in the presence of ouabain (Table IV, K^+ efflux). Under these conditions the Na^+/K^+-pump was blocked, no Na^+ efflux through the $Na^+/K^+/Cl^-$ cotransport could be present and no significant difference was detected in the total K^+ efflux. Other transport systems, such as Na^+/H^+ exchange, are related to cell volume [36]. However, the Na^+/H^+ exchange in RBC is detectable only in conditions of intracellular acidification [37] and no difference between MHS and MNS RBC were found in Na^+/H^+ exchange (Canessa, personal communication). The Na^+/Li^+ exchange, which may represent an operation mode of the Na^+/H^+ antiport, does not seem present in MHS and MNS RBC (Torielli, L., unpublished data). Therefore, the smaller RG volume of MHS rats did not seem influenced by the ion transport systems, but was most likely due to some intrinsic characteristic of the cell membrane structure. Both cell volume and $Na^+/K^+/Cl^-$ cotransport could be under modulation of a common transduction mechanism, for instance a primary deformation of the cell skeleton [38].

The macroscopic protein pattern of RG of both strains, as obtained by SDS-PAGE (Fig. 1), was comparable and consistent with the maintenance of the skeletal membrane component [19,39]. However, as discussed below, a difference in the primary sequence of a membrane skeletal protein between MHS and MNS has been previously demonstrated [40].

$Na^+/K^+/Cl^-$ cotransport in rat RG

Studies by other authors have never been directly orientated toward the characterization of the $Na^+/K^+/Cl^-$ cotransport in RG, so it has not previously been either detected or recognized [25–28].

The following criteria should be fulfilled for the characterization of a Na^+ efflux through the $Na^+/K^+/Cl^-$ cotransport: (a) the K^+- and Cl^--dependence of the Na^+ efflux; (b) the activation by intracellular Na^+ of the K^+- and Cl-dependent Na^+ efflux; (c) the dose-dependent inhibitory activity of a loop diuretic, such as bumetanide, recognized as specific inhibitor of the cell $Na^+/K^+/Cl^-$ cotransport [33]. We chose to measure the Na^+-efflux component of the $Na^+/K^+/Cl^-$ cotransport because: (1) the high K^+ permeability of RG [26] (Table IV) may mask some differences of the $Na^+/K^+/Cl^-$ cotransport between MHS and MNS rats; (2) all the results obtained up to now on the activities of the $Na^+/K^+/Cl^-$ cotransport in intact RBC [10] or IOV [18] of the Milan rats concerned the Na^+ efflux component. Therefore, we wanted to compare RG, RBC and IOV under the same conditions. As reported in Table II, the Na^+ efflux measured in rat RG was inhibited by 80% by the removal of intracellular K^+ and was dependent on the presence of Cl^- ions, since the substitution with two different anions, NO_3^- and SO_4^{2-}, reduced the Na^+ efflux by approx. 60%. Bumetanide inhibits the Na^+ efflux from RG in a dose-dependent way (Fig. 3), giving IC_{50} values (RBC 15 mM, RG 24 mM) in accordance with those reported for rat RBC [7]. The Na^+ efflux was stimulated by increasing intracellular Na^+ concentrations (Fig. 4), as expected for the $Na^+/K^+/Cl^-$ cotransport. All these properties strongly suggest that the bumetanide-sensitive Na^+ efflux measured in rat RG represents the $Na^+/K^+/Cl^-$ cotransport.

To our knowledge, this is the first direct demonstration of the presence of such a cotransport in RG. Other authors studied in detail the characteristics of the K^+/Cl^- cotransport in human RG [25–28], but did not detect a $Na^+/K^+/Cl^-$ cotransport. There may be a few reasons for missing the $Na^+/K^+/Cl^-$ cotransport in those previous studies. O'Neill [26] measured the Cl^--dependent K^+ influx in human RG, resealed and maintained at physiological ion concentrations. Under these conditions, he did not find a stimulatory effect of extracellular Na^+ on K^+ influx, as expected for a $Na^+/K^+/Cl^-$ cotransport but, actually, the presence of Na^+_{out} inhibited the K^+ influx. It has been demonstrated that in intact RBC one mode of operation of the $Na^+/K^+/Cl^-$ cotransport is a K^+/K^+ exchange, that is trans-stimulated by intracellular Na^+ and K^+ only in absence of extracellular Na^+ [41]. It is, therefore, possible that part of the K^+ influx measured in RG by O'Neill in the absence of Na^+_{out} was mediated by the K^+/K^+-exchange operation mode of the $Na^+/K^+/Cl^-$ cotransport and the addition of Na^+_{out} inhibited this pathway or, alternatively, that the enhancement of the total Cl^--dependent K^+ influx obtained by the removal of Na^+_{out} as reported by O'Neill might mask a Na^+-dependent $Na^+/K^+/Cl^-$ cotransport. Dunham et al. [25] measured the furosemide-sensitive net Na^+- and K^+ influx in human RG loaded and suspended in medium at equimolar concentrations of Na^+ and K^+ and compared these unidirectional fluxes with those in intact cells. Actually, the authors reported the presence of a furosemide-sensitive Na^+ uptake in RG, which was highly variable but in the same range of activity as the Na^+ flux of intact cells. However, no focus was made by these authors on this pathway that may represent the $Na^+/K^+/Cl^-$ cotransport. Probably, the high Na^+ and K^+ permeability exhibited by the ghosts prepared under their experimental conditions (31- and 15-fold higher than in intact erythrocytes, respectively) could affect the measurement of other specific carriers such as the $Na^+/K^+/Cl^-$ cotransport.

The reasons explaining our positive results on the identification of a $Na^+/K^+/Cl^-$ cotransport in rat RG can be summarized as follows: (1) the zero-trans method used in our study for measuring the

bumetanide-sensitive Na^+ efflux maximizes the rate of ion translocation allowing a more precise measurement; (2) our RG preparation shows only a 3–4-fold higher Na^+ passive permeability as compared to intact RBC, which reduced the assay variability; (3) it cannot be excluded that for some species-specific mechanism, rat RG retain a $Na^+/K^+/Cl^-$ cotransport otherwise lost in human ghosts.

$Na^+/K^+/Cl^-$ cotransport in RG of MHS and MNS rats

As reported in Table III and Fig. 4, the $Na^+/K^+/Cl^-$ cotransport rate of MHS RG is higher than in normotensive MNS preparations. This result is qualitatively and quantitatively superimposable on that obtained in intact RBC as reported both in this paper (Table III) and in previously published studies [10,18]. The difference between MHS and MNS $Na^+/K^+/Cl^-$ cotransport in RG do not seem dependent on the difference in RG volume (Table II), since the activity of cotransport is reported as a rate constant, the value being normalized both for the intracellular Na^+ concentration and for the influence of ghost volume on ghost packing. The use of rate constant is allowed [42] as the effluxes were measured at Na_i^+ around 3 mmol/l in RBC and 6 mmol/l in RG, well below the $Na^+/K^+/Cl^-$ cotransport K_m in the RBC (MHS 10.3 $\pm$ 0.57 mmol/l RBC, MNS 19.2 $\pm$ 0.87 mmol/l RBC [18]) and in RG (MHS 9.14 mmol/l RG, MNS 15.38 mmol/l RG). The K_m values obtained in intact RBC and RG are in good agreement for each strain, further supporting the higher affinity for Na_i^+ of MHS $Na^+/K^+/Cl^-$ cotransport in RBC [18]. The possibility that a different activation of the K^+ efflux, between MHS and MNS RG, could affect the bumetanide-sensitive Na^+ efflux can be excluded. Both the bumetanide-sensitive K^+ efflux (may be a K^+-Cl^- cotransport [26,28]) and the bumetanide-resistant K^+ efflux (may be a K^+ passive permeability) are not statistically different between MHS and MNS RG, even though the latter tends to be faster in MHS (Table IV). The small difference may affect the measurement of the $Na^+/K^+/Cl^-$ cotransport, but we think that this influence is negligible since the $Na^+/K^+/Cl^-$ cotransport is 44% higher in MHS while the bumetanide-sensitive K^+ efflux is only 11% higher. This data might also be suggestive of the presence in rat RG of a K^+/Cl^- cotransport, similar in the two preparations, and defined as the Na^+-independent, bumetanide-sensitive K^+ efflux. However, we did not further characterize this pathway, since this was beyond the scope of the present study.

The difference in $Na^+/K^+/Cl^-$ cotransport activity between MHS and MNS rats is maintained in RG, leading to the exclusion of an influence of the cytosolic component in determining such a difference in intact cells. We previously demonstrated that the removal of the membrane skeleton from RBC, as obtained with the IOV preparations [18], nullified both the differences in the $Na^+/K^+/Cl^-$ cotransport rate and affinity for internal Na^+ between the two strains. Taken together, the findings on intact RBC, IOV and RG are suggestive of a role played by the membrane skeleton in determining a different modulation of the $Na^+/K^+/Cl^-$ cotransport at the internal Na^+ site in MHS RBC. In an attempt to identify the molecular abnormality underlying such a cell functional alteration we are studying the membrane skeletal components of the RBC membrane in the two strains, with particular attention toward a 110 kDa protein, identified as adducin [43–45], whose gene sequence demonstrated the presence of a point mutation between MHS and MNS [40]. The possibility that such a protein may be involved in the different $Na^+/K^+/Cl^-$ cotransport modulation between MHS and MNS is currently under investigation.

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