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$Na^+/K^+/Cl^-$-cotransporter mediated Rb^+ fluxes in membrane vesicles from kidneys of normotensive and hypertensive rats

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This paper describes experiments to examine Rb^+ fluxes via the $Na^+/K^+/Cl^-$ cotransporter in membrane vesicles from renal outer medulla of three strains of rat: (A) Wistar (B) Milan hypertensive (MHS) and normotensive (MNS), and (C) Sabra salt-sensitive hypertensive (SBH) and salt-resistant (SBN). Initially, Na^+-dependent furosemide- or bumetanide-inhibited $^{86}Rb^+$ fluxes were characterised using Wistar rat microsomes. The latter were partially purified on a metrizamide cushion, and assay conditions were optimized for use with microsomes from the other rats. The major result is that in microsomes from adult Milan hypertensive (MHS) rats the rate of the $Na^+/K^+/Cl^-$-cotransporter mediated ^{86}Rb flux at sub-saturating concentrations of Rb, appears to be significantly greater than in the normotensive (MNS) controls. The effect reflects an increased apparent Rb affinity of the cotransporter in MHS microsomes. There is no difference in maximal rate or in the apparent Na^+ activation affinity of the $^{86}Rb^+$ flux. In addition bumetanide appears to be a somewhat more effective inhibitor in MHS compared to MNS microsomes. The $^{86}Rb^+$ flux result is compatible with a previous finding that in red cells, Na^+/K^+-cotransporter mediated fluxes are increased in MHS compared to MNS. It supports the notion that the $Na^+/K^+/Cl^-$-cotransporter in both red cells and kidney is a genetic marker for hypertension. It is of interest that apparently more than one Na^+ transport system is affected in MHS hypertensive kidneys (a) the $Na^+/K^+/Cl^-$ cotransporter in the thick ascending limb of Henle and (b) the Na^+/H^+ exchanger and/or a conductive Na^+-pathway in brush-border membranes from proximal tubule. It is conceivable that in the hypertensive animals a common regulatory pathway (e.g., phosphorylation) or protein (e.g., cytoskeleton) is affected along the length of the nephron. In Sabra SBH and SBN rat microsomes, no difference was found for the $^{86}Rb^+$ flux via the $Na^+/K^+/Cl^-$ cotransporter (or via a K^+ channel).

Introduction

Studies on genetically hypertensive rats of both spontaneous and salt-sensitive strains indicate the existence of an inherited defect in the ability of the kidney to excrete Na^+ and this precedes appearance of the hypertension [1,2]. In the highly inbred Milan spontaneously hypertensive strain (MHS) and normotensive controls (MNS), where detailed studies of kidney function have been performed at different stages of development, the picture is now fairly clear. In the pre-hypertensive stage the whole kidney glomerular filtration rate in MHS is higher that in MNS controls, while renal Na^+ excretion is similar and thus tubular Na^+ re-absorption is faster in MHS than MNS [2–4]. In another animal model, the Sabra salt-sensitive hypertensive strain (SBH) [5], a strikingly impaired ability to excrete a saline load has been observed compared to the salt-resistant strain (SBN), i.e., the SBH rats retain Na^+ prior to exposure to the salt/deoxycorticosterone regime which induces the hypertension [6].

It is therefore a major challenge to identify inherited defects in membrane transport of Na^+ and relate these to development of hypertension. Many studies of Na^+ transport in red cells of both essential hypertensive humans and genetically hypertensive rats have now been reported (see, for example, Refs. 7–9). For humans, the pattern of differences in Na^+ transport between normotensives and hypertensives is complicated by the heterogeneity of the hypertensive population [10]. One or more of the following systems may show

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difference: Na^+/K^+ pump; Na^+/K^+ cotransporter; Na^+/Li^+ exchange or passive Na leak. By contrast the inbred MHS strain shows a clearly enhanced outward Na^+/K^+-cotransport flux compared to normotensive (MNS) controls in the pre-hypertensive and adult stage [11]. The MHS red cells are smaller then MNS cells and the cell Na^+ is lower [11]. The differences are genetically related to the hypertension as shown in red cells from F_2-hybrids obtained by crossing F_1 (MHS × MNS) hybrids [12] and from animals after bone marrow transplantation [13]. In red cells from hypertensive SBH rat it is the passive Na^+ leak which appears to be greater than in the SBN salt-resistant strain [8].

By comparison with red cells little is known as to differences in renal transport pathways between normotensive and hypertensive rats. It is known that kidneys of MHS rats are smaller than MNS rats [2]. As in red cells the proximal tubule cells in MHS are smaller than in MNS [27] and cell Na^+ is also lower in MHS compared to MHS [14]. Recent studies using isolated brush-border membrane vesicles (BBMV) from renal cortex show an increased Na^+ flux in MHS compared to MNS [15,16] via the Na^+/H^+ exchanger and/or a conductive Na^+ pathway. This finding is consistent with the evidence for an increased Na^+ re-absorption in MHS nephron [16]. Na^+/glucose cotransport is apparently unaffected in MHS rats [15]. However, the question remains whether the Na^+-transport abnormality is restricted to the proximal tubule. Another important question is whether the abnormality in the red cell Na^+/K^+ cotransport flux is indeed a 'marker' for hypertension, and is found also in the MHS kidney. In the renal tubule the $Na^+/K^+/Cl^-$ cotransporter is restricted to the luminal surface of the cells of the thick ascending limb of the loop of Henle (TALH), the site of action of furosemide and bumetanide [17].

The object of the present study is to attempt to address these questions by looking at the diuretic-inhibited fluxes of $^{86}Rb^+$ in membrane vesicles prepared from the red outer medulla of MHS/MNS and also SBH/SBN rat kidneys. This utilizes a technique developed previously to look at the $Na^+/K^+/Cl^-$-cotransporter mediated $^{86}Rb^+$ flux in partially purified luminal vesicles from rabbit renal outer medulla, i.e., enriched in membranes from the TALH segment of the nephron [18].

Methods

Kidneys were obtained from the following strains of rats, usually 4–6 animals per experiment:

(A) Wistar: Male 3–4 months old and approx. 250 g weight. (Weizmann Institute Hormone Research Department Animal House).

(B) Milan hypertensive (MHS) or normotensive (MNS): Adult rats approximately 2 months old and 200 g weight. Systolic blood pressure (MHS) 162 ± 2.4 mmHg; MNS 127 ± 3.5 mmHg. (Farmitalia Carlo Erba Research Institute, Nerviano).

(C) Sabra salt-sensitive hypertensive (SBH) and salt-resistant (SBN): male 3.5–4 months old and 200–250 g weight. Blood pressures SBH ≈ 140 mmHg; SBN ≈ 120 mmHg. (Hadassah Medical School, Hebrew University).

Systolic blood pressure was measured at the tail using an air cuff connected to a W-WBP Recorder. The animals were pre-warmed at 37 °C for 20 min.

Preparation of membrane vesicles from kidney medulla (see Refs. 18,19)

Outer medulla of kidneys from 4–6 animals of each strain were excised and homogenized in 30 vol. of homogenization buffer: sucrose, 170 mM; KCl, 75 mM; Mops neutralized with triethanolamine (TEA), 10 mM (pH 7.2); EGTA (TEA), 1 mM; $MgCl_2$, 2 mM, supplemented with 4 mM ATP (Tris) (total osmolarity = 363 mosmolar). Based on previous experience with rabit microsomes [18], ATP was added to the medium, but it is not known whether addition of ATP is beneficial in the case of rat microsomes. The homogenate was centrifuged at 7000 rpm for 15 min (Sorvall, SS34) and the supernatant was collected and centrifuged at 20000 rpm for 45 min. The pellet was resuspended in 1 ml of homogenization buffer without ATP, at 3–5 mg protein/ml. The crude microsomes were either used immediately or stored overnight at 4 °C. In the next step 0.5–1.0 ml of crude membrane suspension (2–4 mg protein) was layered on a two-step metrizamide cushion (1 ml of 30% + 7 ml of 15% w/v dissolved in homogenization buffer) and was centrifuged in a Beckman ultracentrifuge, SW41 swing out rotor, at 24000 rpm for 2.5 h. Heavy or 'leaky' vesicles sedimented to the 30%/15% interface. 900 μl of the upper sample layer containing the light vesicles, i.e., impermeant to metrizamide, were collected from each tube and used for transport experiments. The recovery of the relatively purified membranes was calculated to be 20–30% of the crude membrane protein applied. Protein was determined by the method of Bradford [20] using bovine serum albumin (BSA) as standard.

Transport assays

The transport assays are similar to those described previously, [18] with some minor changes to improve reproducibility. Typically 400 μl of metrizamide cushion vesicles (0.2 mg) protein was first mixed with 40 μl of a solution (OVM) containing ouabain 10 mM; vanadate, 1 mM and $MgCl_2$, 10 mM and incubated at room temperature for 30 min. For crude microsomes the volumes used would be 50–100 μl microsomes (0.2–0.4 mg) and 5–10 μl of the OVM solution. 5–6 cm columns of Dowex 50-X8 (50–100 mesh) - triethanolamine form, in Pasteur Pipettes, were washed

with 1 ml of sucrose solution 380 mM containing BSA 25 mg/ml. Then external K^+ was replaced with TEA by applying about 200 μl of metrizamide cushion microsomes (*c* 0.1 mg protein) or 50 μl crude microsomes to a Dowex column, and eluting the vesicles with 1.5 ml of ice-cold sucrose, 380 mM. If desirable, the first 400 μl of eluate could be discarded since the dead space of the column is approx. 600 μl. Good reproducibility in transport assays was achieved if sets of microsomes were applied to and eluted in series from the same Dowex column and so this procedure was used when sets of hypertensive and/or normotensive microsomes were to be compared. Prior to each application of microsomes the Dowex column was washed with 2 ml of sucrose 380 mM. $BaCl_2$, 4 mM was added to eluted vesicles except where $^{86}Rb^+$ fluxes via the K^+ channel were to be measured (see legend to Table II). Aliquots of the vesicles were incubated for 5 min at 4°C with furosemide or bumetanide added from freshly prepared solutions (20 mM in TEA, pH 7.2).

For time-course experiments equal volumes of vesicles (about 200–300 μl) and a standard reaction mixture at room temperature were mixed. The reaction mixture consisted of sucrose 220 mM; Mops (TEA), 20 mM (pH 7.2); NaCl, 100 mM; RbCl 0.4–0.8 mM containing about 50 μCi of ^{86}Rb/ml, so that the composition after mixing was sucrose, 300 mM; NaCl, 50 mM; TEA-Cl about 12 mM; RbCl and ^{86}Rb 0.2–0.4 mM; Mops (TEA), 10 mM (pH 7.2) (osmolarity = 365 mosmolar); and furosemide, 0.7 mM or bumetanide, 1 mM. At times indicated in figures 70–100 μl of vesicles were transferred to Dowex columns placed in counting vials and the vesicles were eluted with 1 ml or 1.5 ml of sucrose 380 mM. For flux experiments over a fixed time (2–3 min) equal volumes (100–150 μl) of vesicles, eluted from the first Dowex column, were mixed in triplicate, in a reduction medium without or with diuretic and at the appropriate time aliquots were transferred to the second Dowex column. $^{86}Rb^+$ was measured by Cerenkov radiation. Rates of Rb^+ uptake were calculated from time courses by linear regression.

Calculations

Kinetic parameters for hyberbolic and sigmoid curves were calculated with an IBM personal computer using a non-linear least-squares procedure - ENZFITTER Elsevier-Biosoft.

Results

Wistar rats

Initially control experiments were performed with Wistar rat membranes in order to ascertain that the $^{86}Rb^+$ flux assayed developed for use with rabbit kidney membranes is applicable. Also since it was expected that the microsomes from rat outer medulla comprise a highly heterogeneous population, some effort was investigated to partially purify the vesicles of interest and optimize the assay conditions. Fig. 1 shows representative time courses for $^{86}Rb^+$ uptakes in the presence and absence of furosemide into crude and metrizamide cushion purified microsomes from Wistar rat kidney outer medulla. In Figs. 1A and B, Rb^+ uptake was measured in the standard reaction mixture, while in Fig. 1C NaCl was replaced by choline chloride. In order to demonstrate the degeee of purification on the metrizamide cushion the amount of protein used in Fig. 1B was about one quarter of the crude microsomal protein in Fig. 1A, so that the total uptake of radioactivity was approximately the same. In each case a rapid component of Rb^+ uptake precedes the linear portion. The initial component is not sensitive to furosemide and is therefore of no interest. It probably represents rapid passive equilibration of isotope into a population of

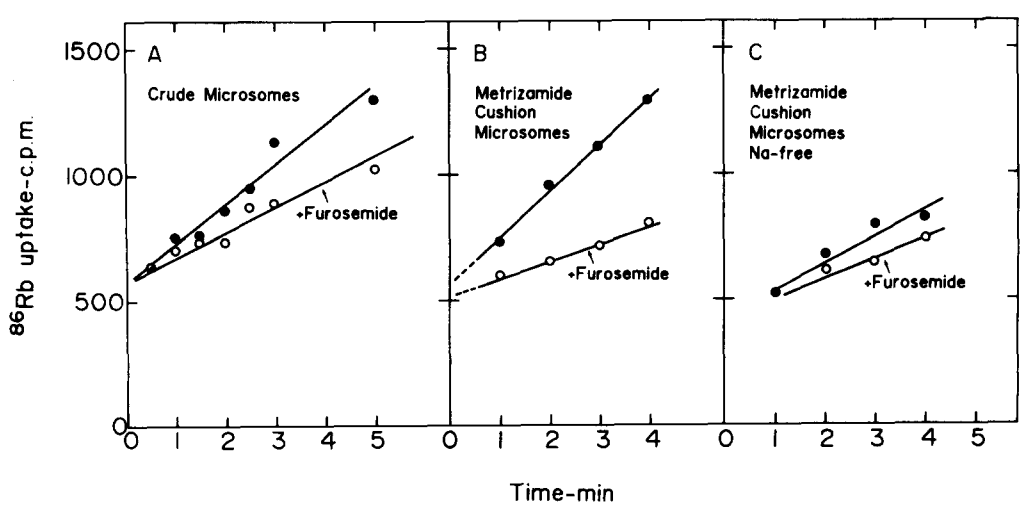

Fig. 1. Time-course of furosemide-sensitive Rb^+ uptake into microsomes from Wistar rats renal outer medulla. Conditions of measurement were described in Methods. The lines drawn represent the best fit by linear regression through all time points with the exception of zero time.

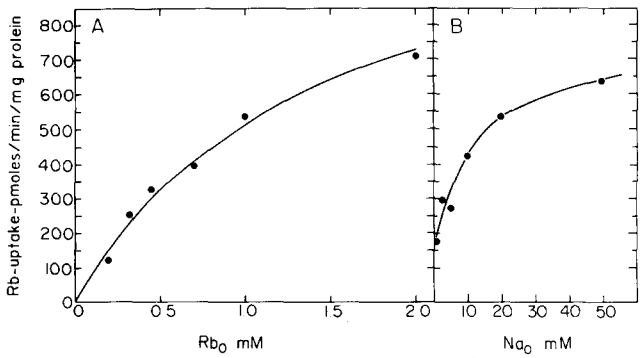

Fig. 2. Furosemide-sensitive Rb^+ uptake at different Rb^+ and Na^+ concentrations. (A) Rb^+ uptake was measured as in Methods in duplicate over 2 minutes in the absence and presence of furosemide, 0.5 mM and a fixed Na^+ concentration of 20 mM. The curve represents the theoretical line using the calculated best fit parameters $V = 1250[Rb]/([Rb] + 1.42)$. (B) Rb^+ uptake was measured as in Fig. 2A at a fixed Rb^+ concentration of 0.5 mM. The osmolality in the external medium was maintained constant by adding choline chloride.

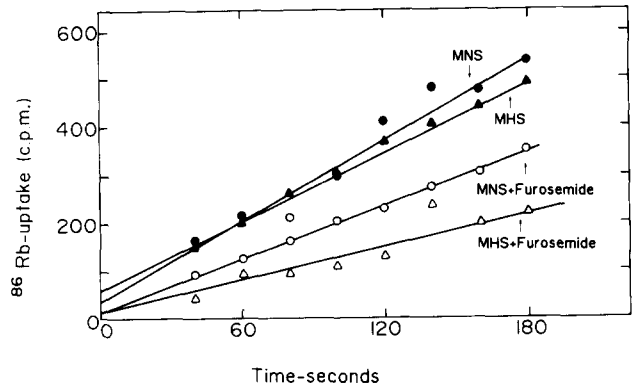

Fig. 3. Time-course of furosemide-sensitive $^{86}Rb^+$ uptake into microsomes from adult MHS and MNS renal outer medulla. Conditions were as in Methods. ●, MNS; ○, MNS + furosemide 0.8 mM; ▲, MHS; △, MHS + furosemide 0.8 mM.

'leaky' vesicles. The specific furosemide-sensitive flux relative to the furosemide-insensitive flux is significantly greater in Fig. 1B than in Fig. 1A. The rates of furosemide-sensitive Rb^+ uptake measured from the linear phase were 49.5 pmol/mg per min in crude microsomes (Fig. 1A) and 179.8 pmol/mg per min in the cushion microsomes (Fig. 1B), demonstrating a characteristic 3–4-fold purification. This purification is due mainly to removal of mitochondral protein and leaky vesicles [19]. The vesicles in Fig. 1B are a mixture of luminal and basolateral membranes since Na^+/K^+-ATPase is purified to about the same extent as transport systems in the luminal surface. On a continuous metrizamide gradient a partial separation of luminal and basolateral membranes can be achieved [18], but this is inconvenient and unnecessary for routine purposes. Fig. 1C shows that in the absence of Na^+, very little furosemide-sensitive Rb^+ uptake is observed. Thus the furosemide-sensitive $^{86}Rb^+$ flux is largely Na^+-dependent.

Fig. 2A shows an experiment to look at the Rb^+ concentration dependence of the furosemide-sensitive flux measured over 2 min. A hyperbolic dependence was observed with a fitted $K_{0.5}$ for Rb^+ of 1.42 ± 0.27 mM and V_{max} of 1.25 nmol/mg per min. The complementary experiment, where Na^+ concentration was varied at a fixed Rb^+ concentration, is shown in Fig. 2B. A simple Michaelis-Menten curve was obtained again with a calculated affinity for Na^+ of 5.2 ± 2.9 mM and V_{max} of 0.69 nmol/mg per min (at 0.5 mM Rb^+).

As discussed previously [18] the furosemide-sensitivity, Na^+ stimulation of Rb^+ uptake and purification on the metrizamide cushion identify the flux as being related to the $Na^+/K^+/Cl^-$-cotransport system. The results with the rat microsomes are similar to those observed previously with rabbit kidney microsomes except that in rabbit there appears to be a more substantial Rb^+ flux in the absence of Na^+, and the absolute rate of the flux is 2–3-times higher than in the rats.

Milan normotensive MNS and hypertensive MHS rats

Fig. 3 shows time-courses of $^{86}Rb^+$ uptake at a sub-saturating Rb^+ concentration, into metrizamide cushion microsomes from outer medulla of MNS or MHS kidneys, in the absence or presence of furosemide (0.8 mM). A linear rate of uptake is maintained for at least 3 min, the difference in the slopes with or without the diuretic providing the best measure of the furosemide-sensitive flux. Evidently the degree of inhibition is significantly greater for MHS then MNS microsomes at this sub-saturating Rb^+ concentration. The total $^{86}Rb^+$ uptake appears significantly smaller in MHS compared to MNS microsomes; this is due only to the use of a lower protein concentration for MHS microsomes in this experiments. The specific activity in pmol/min per mg protein for net, furosemide-sensitive

TABLE I

Rates of furosemide-sensitive and furosemide-insensitive $^{86}Rb^+$ uptake into MHS and MNS microsomes

The $^{86}Rb^+$ flux was measured over three minutes in triplicate in the absence and presence of furosemide, 0.8 mM, in the standard medium containing Rb^+, 0.5 mM and Na^+, 20 mM. In each case the protein applied to the first column was estimated and used to calculate the specific activity. These results represent the average figures from ten different experiments ± S.E. The statistical difference between the MHS and MNS was evaluated using Student's *t*-test. The differences between MHS and MNS total ($P < 0.05$) and furosemide-sensitive ($0.05 < P < 0.1$.) Rb^+ uptakes are significant. $^{86}Rb^+$ uptake (pmol/min per mg protein)

MHS			MNS		
Total	+F	ΔF	Total	+F	ΔF
842 ± 42	433 ± 47	401 ± 37	559 ± 94	406 ± 37	316 ± 28

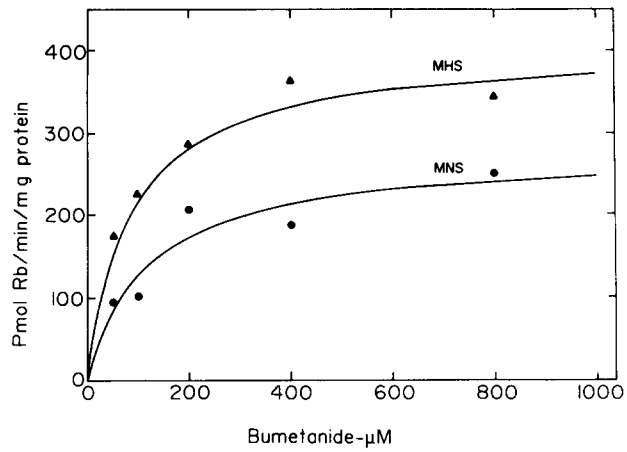

Fig. 4. Sensitivity to bumetanide of $^{86}Rb^+$ uptake into microsomes from MHS and MNS renal outer medulla. Rb^+ flux was measured in duplicate in the standard conditions, over 2 minutes in the absence or presence of the indicated concentration of bumetanide. The curves are the theoretical lines using the following best-fit parameters: ▲, MHS, $V = 406[Bum]/([Bum]+88)$; ●, MNS, $V = 276[Bum]/([Bum]+117)$.

and furosemide-insensitive Rb^+ uptake was determined in the same medium as used in Fig. 1 by measuring the $^{86}Rb^+$ uptake for a fixed time in the absence or presence of furosemide, 0.8 mM. As seen in Table I which presents average results from ten different experiments the total ($P < 0.05$) and furosemide-sensitive ($0.05 < P < 0.1$) Rb^+ uptakes at this sub-saturating Rb^+ concentration are significantly higher in the MHS microsomes, while the furosemide-insensitive Rb^+ uptake is not significantly different for the two species.

Following this initial demonstration of the difference between the two species a systematic comparison of the diuretic-sensitivity of the Rb^+ fluxes was undertaken. These experiments examine furosemide- or bumetanide-inhibited Rb^+ flux in the standard medium for a fixed period of 2 min, over a range of concentrations of the diuretic. Fig. 4 shows a representative experiment using bumetanide. There are two striking features. First, the maximally inhibited flux in MHS is distinctly higher than in MNS microsomes. Second, the apparent affinity for the diuretic is low (K_m MHS, 88 μM; MNS, 117 μM) compared to that expected from experience with whole cells (see, for example, Ref. 21). This low affinity for bumetanide with the rat microsomes is similar to that reported before using rabbit kidney [18] and pig kidney microsomes (unpublished data). It is discussed further below, in relation to the properties of the $Na^+/K^+/Cl^-$ cotransporter in isolated membranes. Table II collects together results from fourteen similar experiments utilizing either furosemide or bumetanide. In each case the diuretic-inhibitable flux is 15–25% higher in MHS compared to MNS microsomes. For both the furosemide and bumetanide-inhibitable flux statistical significance was reached ($P < 0.01$ by two ways analysis of variance). For both the diuretics the calculated affinities are low and are of comparable

TABLE II

Kinetics parameters of the dose-response curves of Rb^+ uptake to furosemide and bumetanide in MHS and MNS TALH vesicle preparation

In these experiments the Rb^+_{out} concentration was 0.5 mM and Na^+_{out} 20 mM. Flux rates and $K_{0.5}$ values were calculated by best-fit analysis using the program ENZFITTER and on IBM Personal Computer. Two ways analysis of variance has been used to evaluate the statistical significance between MHS and MNS preparations. The difference between furosemide-sensitive fluxes is significant at a level $P < 0.01$. The difference between bumetanide-sensitive fluxes is significant at a level $P < 0.05$. The difference in $K_{0.5}$ for bumetanide is significant at a level of $P < 0.01$.

Expt. No.	Diuretic	MHS		MNS	
		diuretic-inhibited Rb^+ flux (pmol/min per mg ± S.E.)	$K_{0.5}$ diuretic (μM ± S.E.)	diuretic-inhibited Rb^+ flux (pmol/min per mg ± S.E.)	$K_{0.5}$ diuretic (μM ± S.E.)
I	Furosemide	495 ± 51	40 ± 21	384 ± 38	77 ± 29
II		310 ± 17	53 ± 13	222 ± 11	25 ± 8
III		541 ± 91	306 ± 103	450 ± 78	355 ± 130
IV		676 ± 38	169 ± 27	537 ± 47	187 ± 44
V		781 ± 104	224 ± 76	601 ± 67	183 ± 55
VI		523 ± 24	115 ± 19	301 ± 12	106 ± 14
M ± S.E.		554 ± 66	151 ± 42	415 ± 58	155 ± 47
VII	Bumetanide	334 ± 54	83 ± 49	271 ± 23	119 ± 33
VIII		406 ± 44	88 ± 34	276 ± 42	117 ± 57
IX		608 ± 43	121 ± 27	586 ± 47	193 ± 31
X		625 ± 56	182 ± 44	582 ± 62	216 ± 35
XI		512 ± 44	127 ± 33	428 ± 83	273 ± 26
XII		381 ± 48	203 ± 54	231 ± 29	225 ± 39
XIII		592 ± 31	223 ± 30	422 ± 63	298 ± 31
X ± S.E.		491 ± 39	145 ± 18	339 ± 48	206 ± 22

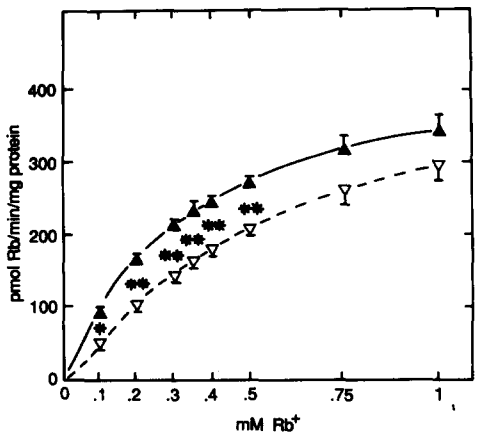

Fig. 5. Bumetanide-sensitive $^{86}Rb^+$ uptake into MHS and MNS microsomes at different Rb^+ concentrations. External Na^+ was kept constant at 20 mM. Other conditions were as in Methods. These curves are the mean ± S.e. of seven separate experiments. ▲, MHS; ▽, MNS; * $P < 0.05$; ** $P < 0.01$.

magnitude. However, bumetanide appeared to show a significantly higher affinity for the transport system in MHS that in MNS ($P < 0.01$).

In order to establish whether the faster diuretic-sensitive Rb^+ uptake of MHS is due to a different affinity of activation by the transported ions (Na^+ and Rb^+), or to an increased maximal transport rate (V_{max}), bumetanide-inhibited Rb^+ uptake was measured in the two strains as a function of different concentration of external Rb^+. Fig. 5 shows the bumetanide sensitive Rb^+ uptake as function of external Rb^+, at fixed external

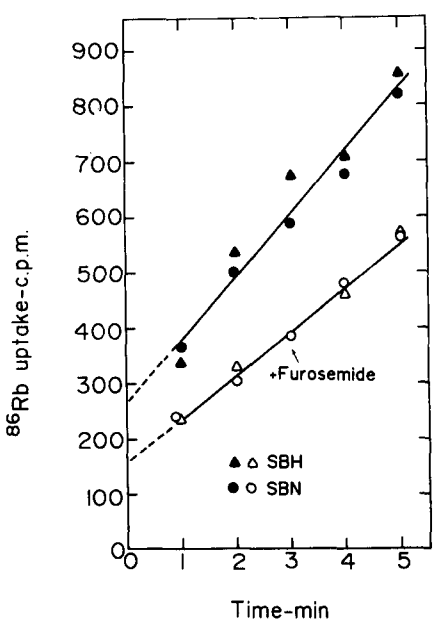

Fig. 6. Time-course of furosemide-sensitive $^{86}Rb^+$ uptake into microsomes from SBH and SBN renal outer medulla. Conditions were as in Methods. ▲, SBH; △, SBH + furosemide; ●, SBN; ○, SBN + furosemide. Lines drawn are the best fit by linear regression using the average values from SBH and SBN for individual values of rates, see Table II.

TABLE III

Rates of Rb^+ uptake via the $Na^+/K^+/Cl^-$ cotransported and a K^+ channel in microsomes from SBH and SBN rat renal outer medulla

Furosemide-sensitive Rb^+ fluxes were measured as described in Methods. The Rb_o^+ concentration in Expt. 1 was 0.4 mM while that in Expt. 2 was 0.2 mM. Flux via the K^+ channel was measured in the absence or presence of Ba^{2+} ions, 2 mM in time-course over five minutes (see also Ref. 19). The Ba^{2+}-inhibited component represents that via the K^+ channel.

Expt.	Furosemide-sensitive Rb^+ uptake (pmol/mg per min)		Ba^{2+}-sensitive Rb^+ uptake (pmol/mg per min)	
	SBH	SBN	SBH	SBN
1. Crude Micr.	81 ±15.4	69 ±15.0	-	-
1. Metrizamide Cushion micr.	316.3±30.0	365.8±24.0	219 ±17.0	180± 9.5
2. Metrizamide Cushion micr.	102.6± 6.2	128.4±12.1	183.1±12.1	211±33.2

Na^+ (20 mM). The Rb^+ activation curves fit a slightly sigmoidal shape for both strains. The kinetic parameters obtained by non-linear regression were as follows: MHS: V_{max} 421 ± 39 pmol/mg per min; $K_{0.5}$ 0.289 ± 0.04 nM; n_H 1.197 ± 0.06. MNS: V_{max} 450 ± 44 pmol/mg per min; $K_{0.5}$ 0.541 ± 0.06 mM; n_H 1.2 ± 0.06 (mean ± S.E. of seven separate experiments). It is evident that the greatest degree of difference between MHS and MNS was observed at non-saturating Rb^+ concentrations, that is the apparent affinity for Rb^+ is higher in MHS that MNS ($P < 0.01$), while the V_{max} is comparable in the two strains.

Sabra salt-sensitive SBH and salt-resistant and SBN rats

Fig. 6 shows time-courses of $^{86}Rb^+$ uptake without or with furosemide into microsomes from SBH and SBN renal outer medulla. Identical quantities of protein were used. There is clearly no significant difference between the two species. Table III summarizes two experiments with SBH and SBN microsomes comparing both furosemide-inhibited $^{86}Rb^+$ and also Ba^{2+}-inhibited $^{86}Rb^+$ uptake via the K^+ channel (see Ref. 19). In no case was any significant difference detectable.

Discussion

The results with Wistar rat microsomes in Fig. 1, namely inhibition of $^{86}Rb^+$ uptake by furosemide, and dependence on Na^+ ions, indicate that this flux is associated with the $Na^+/K^+/Cl^-$ cotransporter. In addition the $K_{0.5}$ for Rb^+ (Fig. 2) and partial purification on the metrizamide gradient (Fig. 1) are similar to observed properties of this flux in rabbit microsomes, where more direct evidence was obtained that the flux is occurring in luminal membranes of the TALH cells [18]. The one surprising feature of the Rb^+ fluxes in the

rabbit microsomes, namely the low apparent affinity for the diuretics furosemide and bumetamide, is found also in all rat microsomes (see, for example, Fig. 3) and pig microsomes (unpublished work of W. Breuer and S.J.D.K.). As reasoned before [18], the low affinity for bumetanide in inhibiting the Rb^+ flux is attributable partly to the fact that the conditions of the assay are sub-optimal for binding, which requires the presence of both Na^+ and K^+ at about 10 mM and low concentrations of Cl^- [22]. However, as suggested previously, the system might have undergone a change in its properties upon isolation of the membranes, which reduces the diuretic affinity. Consistent with this notion we have looked for and failed to detect high affinity bumetanide binding in the MHS and MNS microsomes, in conditions which are known to be optimal for bumetanide binding in other systems [22] (unpublished data). For reasons given in the previous publication [18], and above, it is likely that the Rb^+ fluxes are attributable to the $Na^+/K^+/Cl^-$ cotransporter. Further support for that conclusion comes from recent experiments using rabbit and pig kidney microsomes showing that the Rb^+ flux in the Na^+-containing medium is also dependent on chloride ions (W. Breuer and S.J.D.K., unpublished data.

Milan rats

The experiments in Figs. 3, 4 and Table I shows that the diuretic-inhibited Rb^+ flux is distinctly greater in MHS compared to MNS microsomes. In principle, the higher diuretic-sensitive flux in MHS microsomes could be the result of, a higher density of cotransporters, or a higher turnover rate and/or of a higher Rb^+ affinity. The experiment in Fig. 5 appears to show that the Rb^+ affinity is higher in MHS compared to MNS microsomes, while the density of cotransporters and turnover rate is probably unaltered since no difference in the V_{max} of transport was detectable (Fig. 5). A different Rb^+ affinity in MHS compared to MNS would be consistent with a change in the properties of the cotransporter protein itself. Another difference observed between MHS and MNS was a significantly higher affinity in MHS for inhibition by bumetanide Table I (K_d, μM) MHS 140 ± 18; MNS 206 ± 22, $P < 0.01$). This too might result from the increase in Rb^+ affinity of the cotransporter, since Rb^+ binding is required for bumetanide binding. The lack of difference for the furosemide affinity between MHS and MNS (Table I), might be due to a different ionic requirement for optimal binding of the diuretic in MHS or to the more specific action of bumetanide than furosemide on the in vitro cotransport system of rat [28].

In experiments with rabbit microsomes evidence was obtained that the luminal membrane vesicles which contain both $Na^+/K^+/Cl^-$ cotransporter and a Ca^{2+}-activated K^+ channel are oriented predominantly right-side out [19]. This was deduced from the fact that the K^+ channel in the luminal membrane is activated only by Ca^{2+} within the vesicles and is inhibited by Ba^{2+} in the exterior medium. Similar effects have been observed with the rat (unpublished data), and the conclusion here too is that the vesicles are oriented right-side out. The direction of the increased diuretic-sensitive $Na^+/K^+/Cl^-$ cotransport in the MHS microsomes is therefore such that if this occurred also in the intact tubule it would lead to an increased rate of entry of Na^+, K^+ and Cl^- into the TALH cells and hence Na^+ reabsorption in this segment of the nephron. This might be consistent with the physiological evidence showing an enhanced tubular reabsorption of Na^+ in the pre-hypertensive MHS kidney [3]. It is important to note that in the present experiments the diuretic-sensitive Rb^+ uptake (i.e., the $Na^+/K^+/Cl^-$ cotransport) has been measured in a quite artificial condition especially as concerns the intravesicular ionic composition. While we work in absence of internal Na^+, in physiological conditions the TALH cotransport works at 40–60 mM internal Na^+ and 100–150 mM K^+ (Rb^+) and 30–60 mM external Na^+ and 1–3 mM K^+, respectively [17]. Thus, obviously it would be desirable to look directly at the diuretic-sensitive fluxes in isolated tubule preparations.

It is interesting that apparently more than one transport system can be affected in the hypertensive kidney as seen from the present result and the previous finding using brush border membrane vesicles [15,16]. These include the Na^+/H^+ exchanger and/or a conductive Na^+ pathway in the proximal tubule, as well as the $Na^+/K^+/Cl^-$ cotransporter in the TALH segment. Very recently it has been shown using isolated TALH tubules that Na^+/K^+-ATPase is higher in the MHS compared to MNS cells (Aperia, A., personal communication). No difference is observed in Na^+/K^+-ATPase purified from MHS and MNS kidneys. Also the Na^+/glucose cotransporter activity is not different in MHS and MNS BBMV's. It is interesting to speculate that perhaps the common feature of the Na^+ transport system affected is a defect or abnormality in a regulatory pathway or protein. It has been suggested that the renal $Na^+/K^+/Cl^-$ cotransporter interacts with cytoskeleton components [23], and the activity of this system is known to be highly sensitive to cell volume in different cells [24]. A specific association of Na^+/K^+-ATPase with ankyrin has recently been reported [25]. If the notion of an altered regulatory pathway or protein in the hypertensive animals is correct it may well be that different Na^+ transport systems will be affected in different cells, e.g., red cells, kidney, smooth muscle, etc., depending on the manner of regulation in the normal state.

The other implication of the present finding is that it appears to confirm the conclusion [12] that the abnormality of the $Na^+/K^+/Cl^-$-cotransporter activity

in red cells is indeed a genetic marker of the hypertension.

Sabra rats

The finding here is clear that there is no difference in Rb^+ fluxes via the $Na^+/K^+/Cl^-$ cotransporter, or K^+ channel between the two strains. Previously it has been shown that Na^+/K^+-ATPase is identical in the SBH and SBN rat kidneys using dissected segments from the entire length of the nephron [26]. Thus, at present no evidence from isolated kidney membranes is available which will explain the apparently impaired ability of SBH kidneys to excrete salt. However, since in red cells of SBH rats, the passive Na^+ permeability is higher than in SBN rats [9] the Na^+ permeability of renal membranes should obviously be looked at. This has not yet been done.

SHR versus WKY rats

The work of Mendonca et al. [9] showed that SHR rat erythrocytes have apparently a lower rate of cotransport fluxes than do WKY, while others report a higher rate in the SHR [29]. Similarly, in vascular smooth muscle cells, some workers find a lower rate of diuretic = sensitive fluxes in SHR [30], while others find a higher rate [31]. These ambiguities may be the result of differences in experimental conditions for comparing the cotransporter fluxes. For example it has been shown that the differences between SHR and WKY smooth muscle cells are greatly accentuated in Ca^{2+}-deficient media in which it is thought the cells become permeable to Na^+ and swell [31].

In preliminary experiments using microsomes prepared from renal outer medulla of SHR and WKY, the furosemide-sensitive $^{86}Rb^+$ uptake, measured under the conditions of Table I, was significantly lower in the SHR compared to WKY (Karlish, S.J.D. and Garay, R., unpublished data). This is consistent with the original work of De Mendonca et al. [9] in erythrocytes. However, in view of the conflicting results mentioned above, a systematic comparison of the cotransporter function in well characterised conditions is clearly required, before definite conclusions can be drawn.

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