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Red Cell Ouabain-resistant Na^+ and K^+ Transport in Wistar, Brown Norway and Spontaneously Hypertensive Rats

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Summary

Our previous studies concerning the role of furosemide-resistant cation leaks in genetic hypertension demonstrated that blood pressure of recombinant inbred strains (derived from F2 hybrids of spontaneously hypertensive and normotensive Brown Norway rats) cosegregated with inward Na^+ leak (determined in saline medium) but not with Na^+ efflux (measured in Mg^{2+}-sucrose medium) or with Rb^+ uptake (found in either medium). In the present study the alterations of particular components of ouabain-resistant (OR) Na^+ and K^+ (Rb^+) transport in erythrocytes of spontaneously hypertensive rats (SHR) were analyzed using saline and Na^+-free (Mg^{2+}-sucrose or choline) incubation media. OR Na^+ net uptake was elevated in SHR as compared to both normotensive strains – Brown Norway and Wistar rats. This was mainly due to an increased bumetanide-resistant (BR) Na^+ inward leak. On the other hand, Wistar rats did not differ significantly from SHR in either OR Na^+ efflux or OR Rb^+ uptakes. Major augmentations of BR Na^+ efflux and BR Rb^+ uptake in SHR erythrocytes were seen not only in Mg^{2+}-sucrose medium but also in choline medium. In both Na^+-free media there was a considerable saturable Na^+_i-dependent component of BR Na^+ and Rb^+ fluxes which was more pronounced in SHR than in BN erythrocytes. A great caution is required for the interpretation of the data on "increased passive membrane permeability" obtained in SHR erythrocytes incubated in Na^+-free media because of the presence of this saturable component which seems to be related to incompletely inhibited Na^+-K^+ pump. It can be concluded on the basis of BR fluxes seen in erythrocytes incubated in saline media which probably reflect true cation leaks that passive membrane permeability of SHR erythrocytes is increased for Na^+ but not for $Rb^+(K^+)$.

Key words

Genetic hypertension – Bumetanide-resistant transport – Na^+ leak – Rb^+ leak

Introduction

Increased passive permeability of the erythrocyte membrane for monovalent cations in spontaneously hypertensive rats (SHR) is a frequently described abnormality of ion transport in this strain with genetic hypertension (Postnov *et al.* 1976, Friedman *et al.* 1976, 1977, Wiley *et al.* 1980, De Mendonca *et al.* 1982, Duhm *et al.* 1983, Harris *et al.* 1984, Feig *et al.* 1985, Heller *et al.* 1990, Fujito *et al.* 1991, Orlov *et al.* 1991). Some data were also obtained in Na^+-free media (De Mendonca *et al.* 1982, Heller *et al.* 1990) which enable easy determination of furosemide- or bumetanide-resistant (BR) Na^+ efflux from Na^+-loaded red cells. Other studies (Friedman *et al.* 1977, Harris *et al.* 1984) employed low temperatures to inhibit active transport processes whereas in the remaining experiments ouabain (ranging from 0.2 to 5 mmol/l) and furosemide or bumetanide (up to 1 mmol/l) were used as respective transport inhibitors. Ion transport studies were usually based upon a comparison of SHR with Wistar-Kyoto (WKY) rats although the latter animals need not be the ideal control strain (Kurtz *et al.* 1989, Louis and Howes 1990).

An optimal possibility how to analyze a relationship between ion transport abnormalities and blood pressure in genetic hypertension is to use either F2 hybrids (Bianchi *et al.* 1985, Kotelevtsev *et al.* 1989, Rota *et al.* 1991) or recombinant inbred strains (Bin Talib *et al.* 1992a,b). The only available set of recombinant inbred strains with genetic hypertension

was derived from F2 hybrids of SHR and normotensive Brown Norway (BN) rats (Pravenec *et al.* 1989). Previous studies suggested that SHR erythrocytes exhibited augmented Rb^+ and Na^+ leaks than BN ones when incubated in sodium nitrate or Mg^{2+}-sucrose media (Orlov *et al.* 1991, Bin Talib *et al.* 1992a,b). However, only Na^+ inward leak (saline medium) but not Na^+ efflux (Mg^{2+}-sucrose medium) or Rb^+ leaks (in both media) cosegregated with blood pressure of recombinant inbred strains (Bin Talib *et al.* 1992a,b). This was a reason for the further study of cation leaks in SHR and two normotensive strains (inbred Brown Norway and outbred Wistar rats) with a special respect to the magnitude of Na^+ and Rb^+ residual fluxes occurring in erythrocytes incubated in saline or Na^+-free media.

Methods

Animals and blood sampling

Thirty-two male spontaneously hypertensive rats (SHR), Brown-Norway (BN) and Wistar rats aged 3–4 months were used. All animals were kept under standard conditions (23±1 °C, 12 h light) and offered pellet diet DOS 2b (VELAZ, Prague) with 170 mmol NaCl/kg and tap water *ad libitum*. Blood pressure was determined by a direct puncture of the carotid artery under light ether anesthesia. About 10 ml of blood were withdrawn from the abdominal aorta and anticoagulated by 150 IU heparin. Triplicates of packed cell volume and hemoglobin were determined on the whole blood. Blood was centrifuged for 10 min (4000 x g, 2–4 °C) and plasma, buffy coat and the uppermost red cell layer were aspirated. Three samples of fresh packed erythrocytes were taken from each animal for estimation of the *in vivo* red cell Na^+ and Rb^+ contents. The remaining erythrocytes were divided into equivalent parts that were used for experiments carried out in saline, Mg^{2+}-sucrose or choline media.

Ion transport measurements

The red cell ion transport assays were designed to characterize particular components of ouabain-resistant (OR) Na^+ and K^+ (Rb^+) transport with a special respect to the magnitude of Na^+ and Rb^+ residual fluxes occurring in erythrocytes incubated in saline or Na^+-free media. The dependence of OR transport on extracellular Rb^+ (Rb^+_o) concentration was studied using saline media whereas the relation to intracellular Na^+ (Na^+_i) was examined in Mg^{2+}-sucrose or choline media.

One part of erythrocytes was washed three times with ice-cold saline medium (in mmol/l: NaCl 147, phosphoric acid 2.5, glucose 5, MOPS-TRIS 10, pH 7.4 at 37 °C, 310 mosmol/kg H_2O) and then resuspended (1 : 1) in the same medium. Cell Na^+ content of remaining erythrocytes was modified by the preincubation (1 ml packed cells for 2 h at 37 °C in 50 ml of saline medium) at four different KCl concentrations. K^+-free medium was used to increase cell Na^+ content, a supplementation with 0.4 or 0.8 mmol/l KCl yielded intermediate cell Na^+ elevations and 4 mmol/l KCl maintained cell Na^+ content at *in vivo* levels. Red cells were thereafter washed three times with ten volumes of ice-cold magnesium or choline washing media (in mmol/l: $MgCl_2$ 105 or choline chloride 147, glucose 5, MOPS-TRIS 10, pH 7.4 at 37 °C) and suspended in 1 ml of the same medium. Cell Na^+ contents were determined in triplicate from each cell suspension.

The incubation was started by the addition of 100 µl of cell suspension (about 40 µl of packed erythrocytes) to 1.75 ml of prewarmed (37 °C) incubation media. Red cells with Na^+ contents close to *in vivo* values were studied in saline media containing 2, 5 and 10 mmol/l RbCl. Erythrocytes with modified Na^+ contents were incubated in either Mg^{2+}-sucrose ($MgCl_2$ 70, sucrose 85 mmol/l) or choline chloride media (choline chloride 147 mmol/l). Both incubation media contained (in mmol/l) RbCl 3.5, glucose 5, phosphoric acid 2.5, MOPS-TRIS 10 and were adjusted to pH 7.4 at 37 °C and 310 mosmol/kg H_2O. Ouabain (5 mmol/l) and bumetanide (10 µmol/l) were used as inhibitors of the Na^+-K^+ pump and $Na^+-K^+-Cl^-$ cotransport system, respectively. Red cells were incubated under gentle shaking for 60 min in a thermostated water bath at 37 °C. The incubation was terminated by placing the incubation vessels into an ice bath. This was followed by a centrifugation for 1 min at room temperature (Hettich Micro-Rapid centrifuge, Tuttlingen, FRG). Cell sediments were washed three times with 2 ml ice-cold choline chloride (150 mmol/l) and hemolyzed with 1.25 ml 6 % n-butanol containing 0.1 % CsCl. Na^+ and Rb^+ concentrations were determined in hemolysates by means of atomic absorption spectrophotometry (Varian Techtron, Melbourne, Australia).

Calculation and statistics

Rates of Na^+ and Rb^+ transport were calculated from the changes of cell cation contents occurring over 60 min of incubation. Fractions of OR Na^+ and Rb^+ transport inhibited by 10 µmol/l bumetanide were considered to represent $Na^+-K^+-2Cl^-$ cotransport. Diffusional leaks were defined as the changes in cell Na^+ and Rb^+ contents seen in the presence of both ouabain and bumetanide (residual fluxes).

Red cell cation contents and transport rates (means ± S.E.M) were expressed per 5.2 mmol hemoglobin which refers to an average value of mean cellular hemoglobin content of one litre of rat erythrocytes. The differences among strains were evaluated by one-way analysis of variance followed by a calculation of the least significant differences (Snedecor and Cochran 1968).

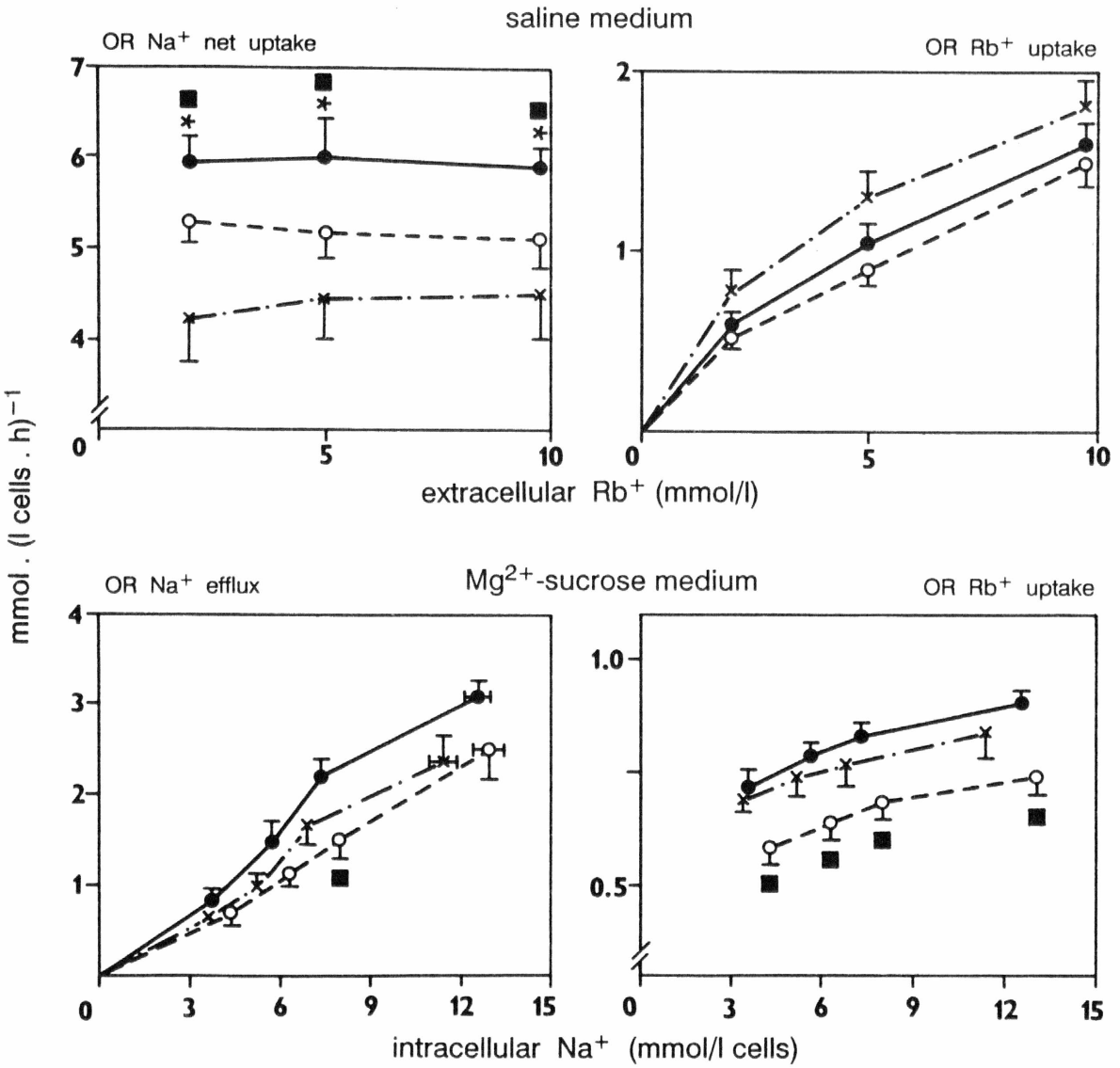

Fig. 1
Ouabain-resistant (OR) Na^+ and Rb^+ transport as a function of extracellular Rb^+ concentration (Rb^+_o) or cell Na^+ content in SHR (dots, full lines), Brown Norway (circles, broken lines) and Wistar (crosses, dashed lines) erythrocytes incubated in saline or Mg^{2+}-sucrose media. Data are means ± S.E.M. from 8 experiments. Asterisks indicate significant differences ($P<0.05$) between SHR and Wistar animals whereas full squares those between SHR and Brown Norway rats.

Results

It should be noted that SHR (MAP 161±8 mm Hg, n=12) had similar red cell Na^+ content (3.17±0.12 mmol Na^+/l cells) as normotensive Wistar rats (119±3 mm Hg, 3.20±0.06 mmol Na^+/l cells, n=8). On the other hand, red cell Na^+ content was significantly elevated in normotensive Brown Norway rats (113±4 mm Hg, 3.76±0.11 mmol Na^+/l cells, n=12).

OR Na^+ net uptake (measured in saline medium) was significantly greater in SHR than in both normotensive strains (BN and Wistar rats) but there were no differences in OR Rb^+ uptake among the strains investigated (Fig. 1). In Mg^{2+}-sucrose medium OR Na^+ efflux and OR Rb^+ uptake were higher in

SHR than in BN rats but Wistar values were close to those of SHR (Fig. 1).

The detailed analysis of particular components of OR ion transport determined in saline medium (Fig. 2) indicated that high bumetanide-resistant Na^+ net uptake (Na^+ leak) was responsible for a great part of the elevation of OR Na^+ net uptake in SHR whereas the alterations of bumetanide-sensitive Na^+ net uptake were not significantly different. BS Rb^+ uptake was greatly elevated in Wistar rats compared to SHR and BN rats whereas no significant differences among strains were disclosed in BR Rb^+ uptake (Fig. 2).

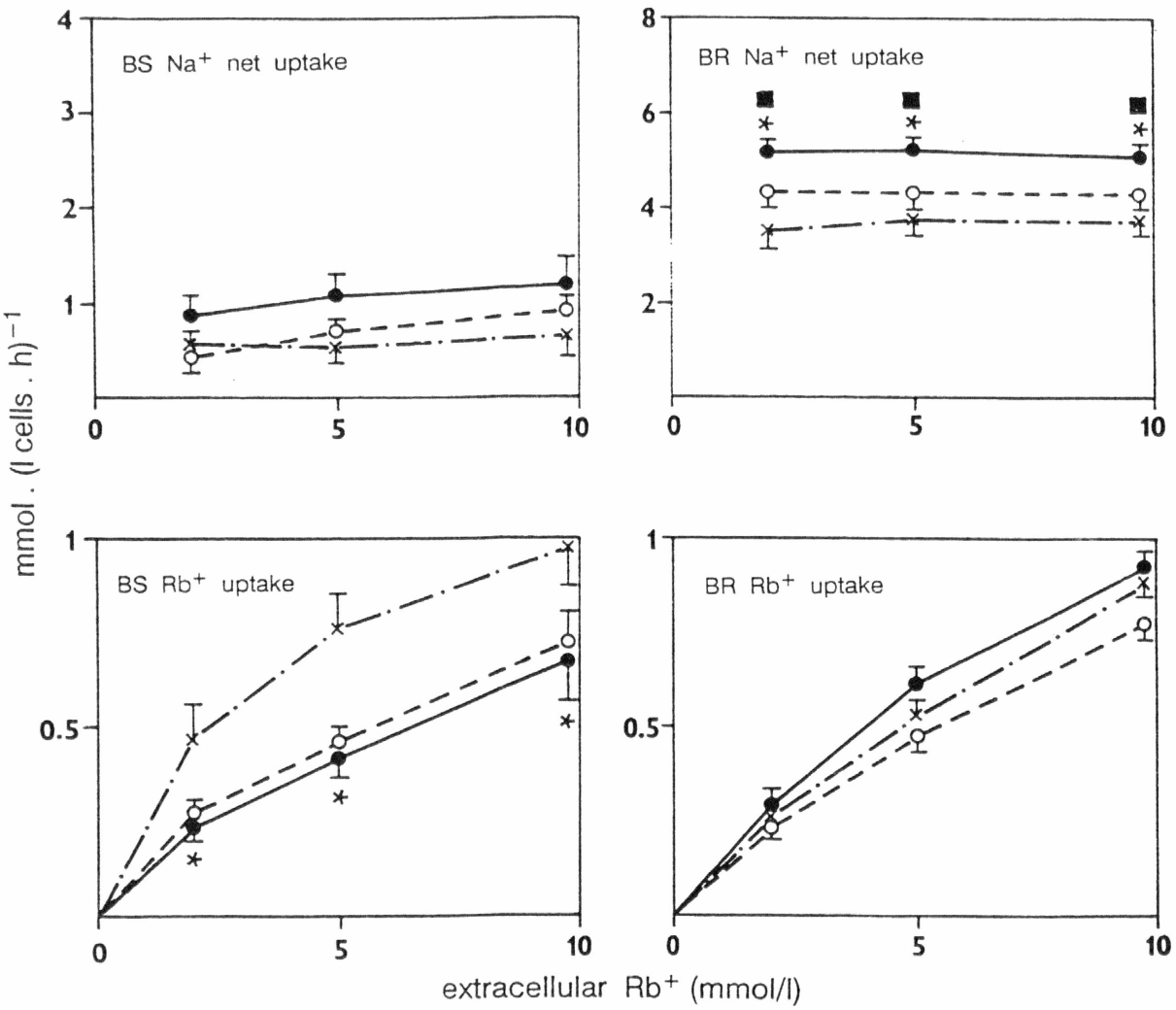

Fig. 2
Bumetanide-sensitive (BS) or -resistant (BR) Na^+ and Rb^+ uptakes as a function of Rb^+_o in SHR (dots, full lines), Brown Norway (circles, broken lines) and Wistar (crosses, dashed lines) erythrocytes incubated in saline medium. Data are means ± S.E.M. from 8 experiments. Asterisks indicate significant differences ($P<0.05$) between SHR and Wistar rats whereas full squares those between SHR and Brown Norway animals.

No significant differences among strains in BS Na^+ efflux or BS Rb^+ uptake were found in erythrocytes incubated in Mg^{2+}-sucrose medium (data not shown). On the other hand, both BR Na^+ efflux and BR Rb^+ uptake were highly elevated in SHR compared to BN rats but Wistar values were close to those of SHR (Fig. 3).

A marked saturable component in BR Na^+ efflux, the dependence of BR Rb^+ uptake on cell Na^+ content and large differences in BR cation fluxes between SHR and BN erythrocytes incubated in Mg^{2+}-sucrose medium required the study of BR ion transport in another Na^+-free medium, i.e. in choline medium. Cation fluxes seen in the presence of both ouabain and bumetanide were generally greater in choline than in

Mg^{2+}-sucrose medium but the difference in BR fluxes between SHR and BN rats was preserved (Fig. 4). Moreover, the non-linearity of BR Na^+ efflux and a considerable dependence of BR Rb^+ uptake on cell Na^+ content (Fig. 4) were also observed in choline medium as in Mg^{2+}-sucrose medium.

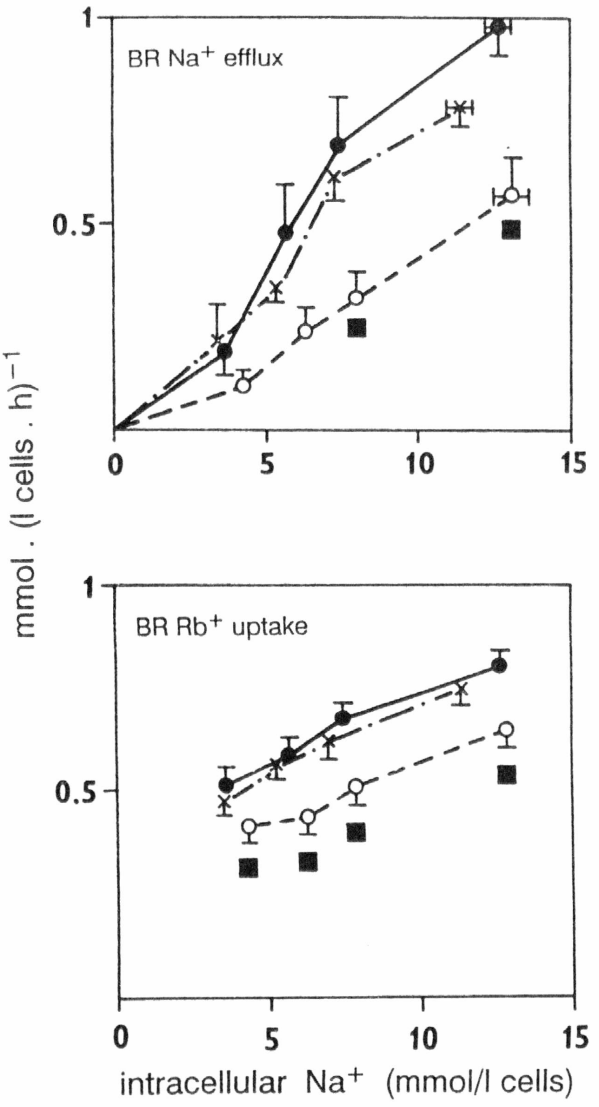

Fig. 3
The relationship of bumetanide-resistant (BR) Na^+ efflux and Rb^+ uptake to cell Na^+ content in SHR (dots, full lines), Brown Norway (circles, broken lines) and Wistar (crosses, dashed lines) erythrocytes incubated in Mg^{2+}-sucrose medium. Data are means ± S.E.M. from 8 experiments. Full squares indicate significantly ($P<0.05$) lower Brown Norway values compared to those seen in SHR and/or Wistar rats.

Discussion

Our present study confirmed the existence of augmented inward Na^+ leak in SHR erythrocytes as compared to those of Brown Norway rats. Increased BR ion fluxes were also observed in Na^+-free (Mg^{2+}-sucrose or choline) incubation media but these data deserve a more detailed discussion.

SHR erythrocytes incubated in saline medium exhibited a substantial elevation of bumetanide-resistant inward Na+ leak but not of BR Rb^+ leak. An apparent contradiction with the previous findings (Duhm *et al.* 1983, Bin Talib *et al.* 1992b) in which significant difference in furosemide-resistant (FR) Rb^+ leak but not in FR inward Na^+ leak was observed, might be explained by the influence of high furosemide concentrations on Na^+ leak and K^+-Cl^- cotransport (Scholz and Hropot 1987, Duhm *et al.* 1990). The augmentation of inward Na^+ leak in SHR was evident not only from a comparison of SHR with inbred BN rats but also with outbred Wistar animals. It is important to note that inward Na^+ leak but not Rb^+ leak cosegregated with blood pressure of recombinant inbred strains derived from F2 hybrids of SHR and BN rats (Bin Talib *et al.* 1992a,b). This was true for both BR and FR Na^+ net uptakes determined in erythrocytes incubated in saline medium (Bin Talib *et al.* 1992a).

On the other hand, the mechanisms underlying augmented BR cation fluxes which were disclosed in SHR erythrocytes incubated in Na^+-free media, need not reflect the same transport phenomena as cation leaks seen in saline medium. Both BR Na^+ efflux and BR Rb+ uptake were enhanced in SHR erythrocytes incubated in either Mg^{2+}-sucrose or choline media but under these conditions Wistar values were close to those of SHR (Fig. 3). This is in a good agreement with our previous findings (Bin Talib *et al.* 1992a,b) that there was no significant association of blood pressure of recombinant inbred strains with either Na^+ efflux or Rb^+ uptake (both BR and FR) determined in Mg^{2+}-sucrose media (Bin Talib *et al.* 1992a).

It is evident from Figs 3 and 4 that BR ion fluxes determined in Na^+-free media comprise a considerable saturable component which is dependent on cell Na^+ content. This component is greater in SHR than in BN rats. It cannot be ascribed to the incomplete inhibition of the $Na^+-K^+-2Cl^-$ cotransport system by a relatively low bumetanide concentration because it is also present in FR ion fluxes (data not shown). This phenomenon concerns both Na^+ and Rb^+ fluxes so that the contribution of an accelerated K^+-Cl^- cotransport in SHR (Rota *et al.* 1991) can also be excluded.

It seems that both BR Na^+ efflux and Rb^+ uptake determined in Na^+-free media might comprise a certain fraction of Na^+-K^+ pump activity.

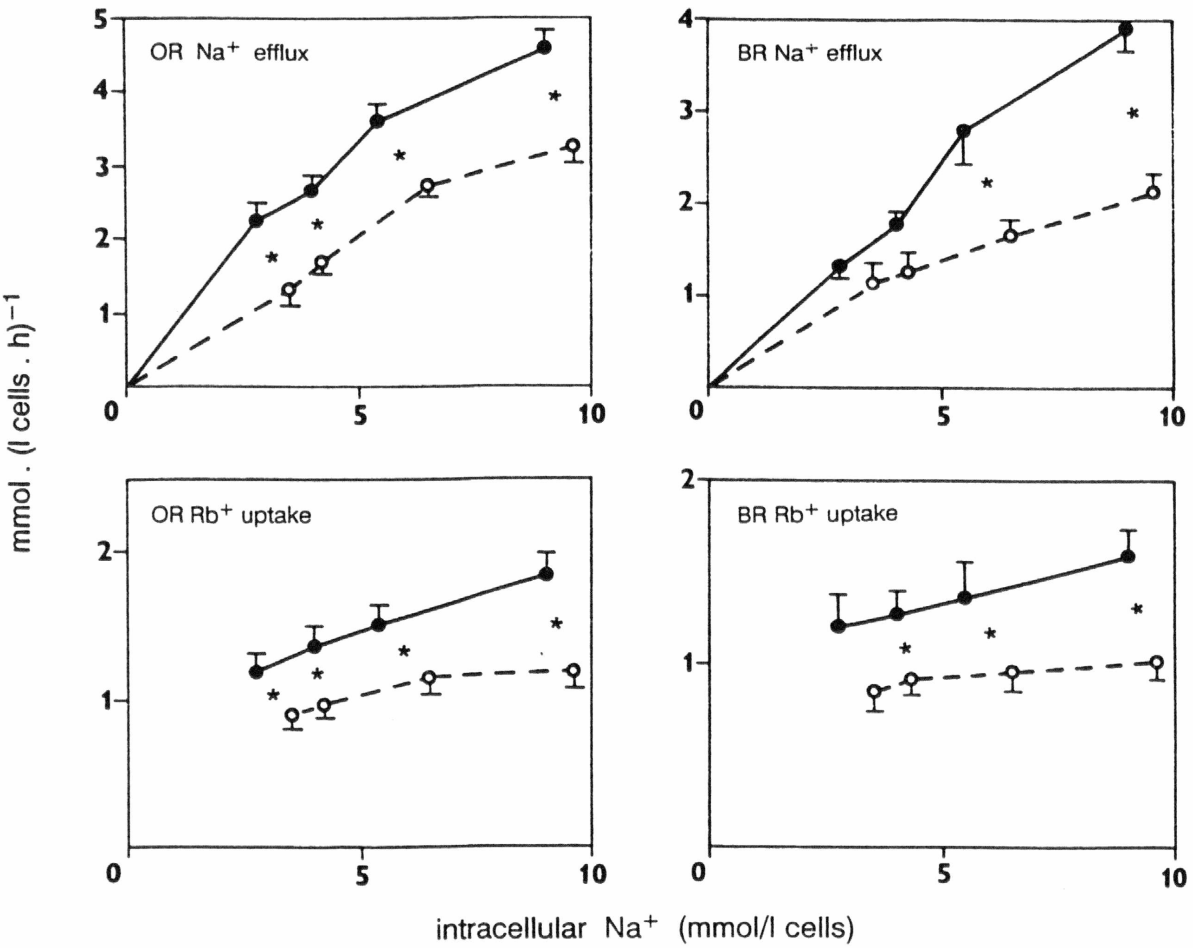

Fig. 4
The relationship of ouabain-resistant (OR) and bumetanide-resistant (BR) Na^+ efflux and Rb^+ uptake to red cell Na^+ content in SHR (dots, full lines) and Brown Norway (circles, broken line) erythrocytes incubated in choline medium. Data are means ± S.E.M. from 4 experiments. Asterisks indicate significant differences ($P<0.05$) between SHR and Brown Norway rats.

Remaining ouabain-sensitive Na^+ extrusion and Rb^+ uptake contribute to a false elevation of respective "leakage" rates. Our recent experiments carried out in Wistar erythrocytes incubated in Na^+-free media indicated that in the absence of extracellular sodium 5 mmol/l ouabain does not inhibit completely the Na^+-K^+ pump. Vanadate (0.2 mmol/l) which is a better Na^+-K^+ pump inhibitor in Na^+-free media than 5 mmol/l ouabain, almost eliminated the saturable component of FR and BR ion transport (Bin Talib and Zicha, unpublished data).

Thus leakage rates determined in saline media might reflect the true changes in passive membrane permeability including those related to genetic hypertension. Of course, this is valid under the assumption that sufficiently high ouabain concentrations (at least 1 mmol/l) are used to inhibit the Na^+-K^+ pump in saline media. On the other hand, a large part of elevated "residual" cation fluxes observed in SHR erythrocytes incubated in Na^+-free media might be caused by the incompletely inhibited Na^+-K^+ pump activity which is known to be higher in SHR than in BN rats (Orlov *et al.* 1991). Consequently, the "residual fluxes" seen in Na^+-free media should be considered with a great caution because they do not represent true cation leaks.

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