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EFFECT OF AMILORIDE ANALOGUES ON SODIUM TRANSPORT IN RENAL BRUSH BORDER MEMBRANE VESICLES FROM MILAN HYPERTENSIVE RATS

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Summary. The effect of ethylisopropyl-amiloride (EIPA) and phenamil on sodium uptake in renal brush border membrane vesicles from prehypertensive rats of the Milan strain (MHS) and their normotensive controls (MNS) was investigated. In the presence of both a membrane potential and a pH gradient a differential effect of EIPA and phenamil was evidenced between the two rat strains. In the absence of a pH gradient, but in the presence of a membrane potential, EIPA was about two-fold more potent than phenamil in inhibiting sodium transport in both rat strains, excluding the presence of epithelial sodium channels in our BBMV preparations. Taken together these results support the hypothesis that a structurally different Na^+/H^+ exchanger located on the brush border membrane may be involved in the increased tubular sodium reabsorption observed *in vivo* in hypertensive rats.

A renal abnormality in sodium handling is involved in the blood pressure difference between MHS and MNS rats (1,2). Studies with brush border membrane vesicles (BBMV) obtained from renal cortical slices of prehypertensive MHS (four-week old) and the age matched MNS have shown that the sodium transport across the luminal membrane of proximal tubule is increased in MHS (3). In the absence of solutes cotransported with the sodium ion by different carriers, the uptake of ^{22}Na in brush border membrane vesicles may be accounted for by a ΔpH-driven mechanism, i.e.

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the Na^+/H^+ antiport (4, 5) and by sodium channels (6). It has been demonstrated that in MHS rats sodium uptake was stimulated by the presence of both a pH gradient and a transmembrane electrical potential difference (3, 7). The uptake was inhibited by amiloride at relatively high concentrations (3), therefore it was not clear which pathway was involved in the difference between the two rat strains. The availaibility of new potent derivatives of amiloride that have a higher affinity for Na^+/H^+ exchange (8, 9) or for the epithelial sodium channel (10, 11) could be useful for distinguishing between these two possibilities (see for a review ref. 12 and 13).

MATERIALS AND METHODS

Brush border membrane preparation and transport assays. A highly enriched brush border membrane preparation was obtained as previously described (3). To measure sodium transport across BBMV from MNS and prehypertensive MHS rats two different procedures were used. For experiments performed in the presence of a pH gradient, sodium uptake was measured at 10 mM $^{22}NaCl$ and 4 s of incubation time as described (3). To measure sodium fluxes through ion-conducting pathways, transport assays were performed at 0.1 mM $^{22}NaCl$ and 15 s of incubation time as described by Garty et al. (14).

Protein concentration was measured according to Bradford (15) using a Bio-rad kit and BSA as standard.

Calculations

Kinetic parameters for dose-response curves were calculated with an IBM personal computer using a non-linear least-squares procedure as described elsewhere (16).

Materials

$^{22}NaCl$ (carrier free) was obtained from Radiochemical Centre (Amersham Int, U.K.); valinomycin from Boehringer (Mannheim, F.R.G.); FCCP from Sigma (St. Louis, MO). 3,5-diamino-6-chloro-N-(diaminoethylene)pyrazine-carboxamide (Amiloride) was a gift from Merck, Sharp and Dohme (GmbH, Munich, FRG); 5-ethylisopropylamino-amiloride and 3,5-diamino-6-chloro-N-((phenylamino)aminomethylene)pyrazine-carboxamide (phenamil) were synthetized as described (17, 18). All other reagents were analytical grade products from Merck (Darmstadt, FRG).

RESULTS

The magnitude of the difference in sodium transport between MHS and MNS depends on sodium concentration and membrane potential ($\Delta\psi$), i.e. on the presence of FCCP (3, 7). At 10 mM NaCl the sodium uptake was 5975 ± 219 and 5090 ± 182 pmol/4s/mg protein, for MHS and MNS respectively ($P < 0.01$, t-test, 14 different preparations).

Table 1. Kinetic parameters of the interaction of amiloride, EIPA and phenamil with ^{22}Na uptake in the presence of ΔpH and an outside positive Δψ. Sodium transport was measured at 10 mM NaCl, in the presence of a pH gradient ($5.5_{in}/7.2_{out}$) and the protonophore FCCP (0.1 μM). The uptakes were subtracted of the nonspecific binding of ^{22}Na to the membranes. Half-maximum inhibition constants (IC_{50}) and Hill coefficients (n) were calculated from dose-response curves with inhibitor concentrations varying from 10^{-6} to 1.7×10^{-3} mol/l; IC_{50} is in μM. Mean ± S.E. of six independent experiments. The statistical significance of the difference was tested by Student's t-test; NS = not significant.

Inhibitor		MNS	MHS	P
Amiloride	IC_{50}	331 ± 41	394 ± 112	NS
	n	1.01 ± 0.12	1.05 ± 0.09	NS
EIPA	IC_{50}	87 ± 14	165 ± 32	< 0.05
	n	0.95 ± 0.12	1.04 ± 0.16	NS
Phenamil	IC_{50}	209 ± 18	138 ± 12	< 0.01
	n	1.35 ± 0.12	1.09 ± 0.09	NS

In order to evaluate half-maximum inhibition by amiloride analogues EIPA and phenamil on the initial rate of 10 mM ^{22}Na uptake, dose-response curves were established for each of them. The concentrations of each derivative that inhibit 50% of the sodium uptake (IC_{50}) are given in Table 1. A simple interaction of the amiloride analogues with the transporter(s) involved in the sodium transport can be inferred, Hill coefficient being in all cases not statistically different from 1. For comparison the IC_{50} values for amiloride obtained in the same experimental conditions are also reported. Changing the uptake conditions by decreasing the external sodium concentration (1 mM) or by preincubating the vesicles with the inhibitors for 60 minutes at room temperature, the structure-activity relationships for the amiloride derivatives assayed did not change (data not shown).

To check the presence in our membrane preparations of the epithelial sodium channels, an experimental protocol able to enhance this transport mechanism was outlined. Specifically, ^{22}Na uptake was measured at very low sodium concentration (0.1 mM NaCl) and in the presence of an inside-negative Δψ imposed across the vesicles by a K^+ electrochemical gradient (50 mM K^+_{in}/< 0.1 mM K^+_{out} plus valinomycin). In these conditions the uptake of 0.1 mM $^{22}NaCl$ was: 275 ± 12 and 229 ± 14 pmol/15s/mg protein for MHS and MNS respectively ($P < 0.02$, t-test, 4 different preparations). In the absence of valinomycin the uptake

Table 2. Kinetic parameters of the interaction of EIPA and phenamil with ^{22}Na uptake in the presence of an outside positive K^+-diffusion potential. Sodium transport was measured in the absence of a pH gradient, at 0.1 mM NaCl and in the presence of an outwardly directed potassium gradient (50 mM K^+_{in}/< 0.1 mM K^+_{out}). Half-maximum inhibition constants (IC_{50}) and Hill coefficients (n) were calculated from dose-response curves with inhibitor concentrations varying from 10^{-6} to 1.7 x 10^{-3} mol/l; IC_{50} is in μM. Mean ± S.E. of four independent experiments. The statistical significance of the difference was tested by Student's t-test; NS = not significant.

Inhibitor		MNS	MHS	P
EIPA	IC_{50} (μM)	80 ± 10	57 ± 8	NS
	n	0.9 ± 0.1	0.8 ± 0.2	NS
Phenamil	IC_{50} (μM)	160 ± 6	140 ± 13	NS
	n	0.8 ± 0.2	0.9 ± 0.2	NS

values were: 111 ± 9 and 86 ± 2 pmol/mg protein for MHS and MNS respectively ($P < 0.05$, t-test, 4 different preparations). The higher uptake rates observed with valinomycin confirmed the presence of a conductive pathway for sodium in BBMV from MHS and MNS rat kidney. To test the sensitivity of this potential-sensitive sodium transport to EIPA and phenamil dose-response curves were established for each of them. Concentrations below 10^{-6} M phenamil or EIPA were uneffective to inhibit sodium uptake. The calculated IC_{50} values for phenamil were higher than that for EIPA in both rat strains (Table 2), suggesting that even in these conditions the Na^+/H^+ antiporter has to be considered as the sole transport mechanism present in BBMV from MHS and MNS rats. Moreover, in these conditions the affinity for the two analogues were not different between the two rat strains.

DISCUSSION

In the presence of an intravesicular acidic pH and an inside negative $\Delta\psi$ sodium uptake in BBMV from MHS and MNS kidney cortex was inhibited by amiloride, EIPA and phenamil (Table 1). However the sensitivity of rat brush border membranes to these molecules seems to be very low in comparison to that of brush border membranes from other species (19) or other cell types (8, 9, 20, 21). The ^{22}Na uptake in these experimental conditions would be accounted for by the Na^+/H^+ exchange mechanism. However, with respect to the blockade of Na^+/H^+ antiporter (8, 19, 21), the structure-activity relationships of the compounds tested were not confirmed. In fact, the following orders of potency were

obtained: EIPA ≃ phenamil > amiloride and EIPA > phenamil > amiloride for hypertensive and normotensive rats respectively (Table 1). A different pattern of inhibition between MHS and MNS seems to indicate a different binding interaction, which in turn may suggest a different conformation or structure of the membrane protein responsible for the binding of these ligands and involved in the sodium uptake by the brush border membrane of MHS rat kidney. Alternatively, differences in distribution of Na^+/H^+ exchange and sodium channel transport pathways between the two rat strains may be responsible of the increased sodium transport in MHS.

The presence of sodium channels in epithelia can be easily detected by using vesicles prepared and assayed with the procedure described by Garty et al. (14). According to this procedure large, rheogenic, amiloride-sensitive sodium fluxes have been demonstrated in tight epithelia (22, 23) and more recently in vesicles prepared from pars recta of rabbit proximal tubules (24). To investigate the existence of ion-conducting pathways in BBMV from MHS and MNS rats, the effect of phenamil and EIPA on sodium transport in the presence of an inside-negative $\Delta\psi$ generated by a K^+ diffusion potential was measured. Using this experimental procedure, that is known to reproduce favourable conditions for optimal binding of the inhibitors to epithelial sodium channels (23), phenamil failed to produce higher inhibitory effect than EIPA (Table 2). The high specifity of phenamil for the epithelial sodium channel is well-documented (11, 12, 13). The IC_{50} of phenamil ranges from 2×10^{-10} to 10^{-7} mol/l in many species and tissues (11, 25). By contrast, phenamil shows a very low affinity for the Na^+/H^+ exchanger, its IC_{50} values are higher than 10^{-4} mol/l (12, 19). Few data are available on rat kidney, but also in this species the substitution of the guanidino group of amiloride with benzenil rings selectively decreases the half-maximum inhibition of the sodium channel (26). If we assume that phenamil effectively functions as a molecular probe for the identification of epithelial sodium channels in our preparation, then a much lower affinity constant than that found by us would be expected. Although a second family of epithelial sodium channels with a low affinity for phenamil (IC_{50} = 10 μM) has been hypothesized in kidney membranes (11), and sodium channels with a low affinity for amiloride have been identified in the apical membrane of the

late proximal tubule in rabbit (6), the ability of EIPA to exert an inhibitory effect higher than that of phenamil on sodium uptake does not agree with the presence of sodium channels in BBMV from MHS and MNS rats. Therefore, in the absence of cotransported solutes, the Na^+/H^+ exchange seems to be the sole entry mechanism for sodium uptake in our membrane preparation. Hence, the $\Delta\psi$-stimulated sodium transport, as shown by its higher sensitivity to EIPA, even though measured in the absence of a pH gradient, did represent the uptake through the Na^+/H^+ antiporter.

In conclusion, the results presented here can be summarized as follows: i) the increased sodium transport in the proximal tubule of MHS rats is mediated by a $\Delta\psi$-sensitive pH-dependent sodium pathway undistinguishable from the Na^+/H^+ antiporter; ii) the differential effect of phenamil, EIPA and amiloride on MHS and MNS rats suggests the presence of structural alterations in the Na^+/H^+ antiporter of MHS; iii) the pharmacological value of these observations has to be considered as premature since relatively high doses of amiloride analogues would be required to inhibit sodium reabsorption in these rats *in vivo*. However a more detailed analysis of the amiloride series might reveal molecular structures endowed with higher selectivity for hypertensive rats.

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