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Isolation of renal brush-border membrane vesicles by a low-speed centrifugation; effect of sex hormones on Na^+-H^+ exchange in rat and mouse kidney

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Na^+-H^+ exchange in rat and mouse renal brush-border membrane vesicles was studied by fluorescence quenching of the ΔpH indicator, acridine orange. Brush-border membrane vesicles were isolated by a modified Mg/EGTA-precipitation method at low speed centrifugation (8000 × *g*). The enzymatic characteristics of these membrane vesicles were similar to those obtained by the original high-speed centrifugation method (Biber et al. (1981) Biochim. Biophys. Acta, 647, 169–176). The rates of Na^+-H^+ exchange in renal brush-border membrane vesicles from male and female rats were similar. Neither ovariectomy nor treatment of ovariectomized rats with estradiol or testosterone changed the activity of Na^+-H^+ exchanger. The rates of Na^+-H^+ exchange in the mouse were smaller than in the rat indicating the existence of species differences. Na^+-H^+ exchange in mouse renal brush-border membranes exhibit strong sex differences, the rates in the male being higher than in the female. Castration of male mice led to a decrease in Na^+-H^+ exchange to values found in females. Treatment of castrated mice with estradiol had no effect. In contrast, treatment with testosterone increased the rate of the exchanger by more than 100%. The effect of testosterone was restricted to the V_{max} of the Na^+-H^+ exchanger, whereas the apparent K_m for Na^+ remained unchanged. Na^+-dependent D-glucose transport in mouse renal luminal membranes exhibited also sex differences due to the potent stimulatory effect of testosterone. Therefore, Na^+-H^+ exchange and Na^+-dependent D-glucose transport in the mouse kidney are under control of androgen hormones. This effect could be in close connection with the wellknown renotropic action of androgens in the mouse.

Introduction

An electroneutral Na^+-H^+ exchange seems to be the major mechanism responsible for H^+ secretion and HCO_3^- reabsorption in the mammalian proximal tubule (for review, see Ref. 1). Recent findings have shown that metabolic acidosis [2], parathyroidectomy [2], uremia [3], unilateral nephrectomy [4], diabetes [5], chronic K^+ depletion [6], and treatment with glucocorticoids [7,8] and thyroid hormones [9] increase the rate of Na^+-H^+ exchange in renal luminal membrane vesicles isolated from various experimental animals. A decrease in renal Na^+-H^+ exchange was observed in metabolic alkalosis [10], hyperparathyroidism [11], and following treatment of diabetic animals with insulin [5]. Thus, Na^+-H^+ exchange in renal proximal tubule can adapt to various physiological and pathophysiological conditions.

Sex steroids are known to affect a number of biochemical and physiological events in cellular membranes of the target tissues [12]. From this

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point of view, a potent renotropic effect of androgens in the mouse kidney could be particularly interesting. Koenig et al. [13,14] have found that testosterone induces a rapid stimulation of calcium fluxes, endocytosis and uptake of amino acids and hexoses into mouse kidney cortex slices. The testosterone effect seems to be mediated by aliphatic polyamines such as spermin, spermidin and putrescin [15] which have been recently found to stimulate D-glucose transport in rabbit renal proximal tubular brush-border membrane vesicles in vitro [16].

In this paper, we describe an easy and unexpensive low-speed centrifugation method for isolation of luminal membrane vesicles from renal proximal tubules. In such membranes prepared from mice and rats we studied species and sex differences as well as an effect of sex hormones, administered in vivo, on Na^+-H^+ exchange.

Material and Methods

Animals and treatment. Experiments were carried out on Wistar rats and BALB/c mice of both sexes at an age of 3 to 4 months. The animals were bred at the Department of Physiology, Faculty of Medicine, in Zagreb. Throughout the experiments, the animals were supplied with pelleted food (Sljeme, Zagreb) and tap water ad libitum.

Female rats were ovariectomized under light ether narcosis by dorsal approach at the age of 4–5 weeks. Two months later, the rats were used in the experiments.

Castration in male mice was performed by the scrotal route under ether anaesthesia in animals aged 5–6 weeks. Experiments on these animals were started about 7 weeks later.

The hormones were dissolved in olive oil and were given intramuscularly in a single dose 8 days before sacrifice. Ovariectomized rats were treated with estrogen or testosterone at a dose of 20 mg per kg body wt. Intact or castrated mice received 2 mg of estrogen or 5 mg of testosterone per animal. Control animals were injected with the equivalent amount of oil (0.2 to 0.5 ml).

Buffers. The following buffers were used throughout the experiments. Buffer 1 (300 mM mannitol/5 mM EGTA/12 mM Tris-HCl (pH 7.4); buffer 2 (150 mM mannitol/2.5 mM EGTA/6 mM Tris-HCl (pH 7.4); Na^+-buffer (300 mM mannitol/150 mM sodium gluconate/5 mM Hepes-Tris (pH 7.0); TMA^+-buffer (300 mM mannitol/150 mM tetramethylammonium gluconate/5 mM Hepes-Tris (pH 7.0); Na^+/K^+-buffer (200 mM mannitol/50 mM potassium gluconate/150 mM sodium gluconate/5 mM Hepes-Tris (pH 7.0); TMA^+/K^+-buffer (200 mM mannitol/50 mM potassium gluconate/150 mM tetramethylammonium gluconate/5 mM Hepes-Tris (pH 7.0); and K^+-buffer (300 mM mannitol/150 mM potassium gluconate/5 Hepes-Tris (pH 7.0).

Isolation of brush-border membrane vesicles. Renal luminal membrane vesicles were isolated by a modification of the Mg/EGTA-precipitation method of Biber et al. [17]. All steps were performed in the cold by using an ice bath, a refrigerated centrifuge (Janetzky K 60) and by placing the centrifuge (microcentrifuge Eppendorf 3200) in an refrigerator.

The rats and mice were killed by a blow on the neck and cervical dislocation, respectively. The kidneys were taken out and immediately immersed into an ice-cold Ringer solution. Cortical slices were dissected with a razor balde. Kidney cortex from one rat or three or four mice was homogenized in 5 ml buffer 1 with an Ultra-Turrax operated at 180 V (1 min homogenization – 2 min pause – 1 min homogenization). 7 ml bi-distilled water and $MgCl_2$ to a final concentration of 12 mM were added to the homogenate. After being kept in an ice bath for 15 min, the homogenate was centrifuged at $2400 \times g$ for 15 min. The pellet was discarded. The supernatant was divided into Eppendorf reaction tubes and centrifuged in an Eppendorf 3200 microcentrifuge ($8000 \times g$) for 20 min. The superantant was removed by suction. The pellet was resuspended in 5 ml buffer 2 with a glass/Teflon homogenizer by 10 strokes at 1000 rpm and $MgCl_2$ was added to yield 12 mM final concentration. Following 15 min incubation on ice the suspension was centrifuged at $2400 \times g$ for 15 min. The pellet was discarded. The supernatant was centrifuged at $8000 \times g$ for 20 min (Eppendorf 3200 microcentrifuge). The resulting supernatant was sucked off. The pellet, which contained brush-border membranes was resuspended in 5 ml of either Na^+-, Na^+/K^+-, or K^+-buffer. The suspension was kept in an ice bath for 30 min

followed by a centrifugation at $8000 \times g$ for 20 min (Eppendorf 3200 microcentrifuge). The resulting dense pellet was suspended with either Na^+-, Na^+/K^+-, or K^+-buffer to the protein concentration of 10 mg/ml.

Protein and enzyme determination. The proteins were determined by the method of Bradford [18] using bovine serum albumin as a standard. Leucine arylamidase (EC 3.4.1.2), acid phosphatase (EC 3.1.3.2) and acetyl cholinesterase (EC 3.1.1.7) activities were measured by using commercial kits (Merckotest No. 3359, Merckotest No. 3305 and Monotest No. 124125, respectively). The activity of $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was measured as described by Berner and Kinne [19], succinate-cytochrome *c* oxidoreductase (EC 1.3.99.1) according to Fleischer and Fleischer [20] and KCN-resistant NADH oxidoreductase (EC 1.6.99.2) according to Sottocasa et al. [21]. Alcohol dehydrogenase activity (EC 1.1.1.1) was determined in the supernatant obtained from the first $8000 \times g$ centrifugation and was measured at room temperature as described previously [22].

Measurement of Na^+-H^+ exchange. Na^+-H^+ exchange in isolated vesicles was measured in both directions, i.e. by monitoring intravesicular acidification in response to an outwardly directed Na^+ gradient (ΔpH developing method) and by measuring the dissipation of a preset ΔpH by an inwardly directed Na^+ gradient. In both cases the changes in transmembrane ΔpH were visualized by the fluorescence quenching of acridine orange [23–26].

For the ΔpH developing method, the vesicles were preloaded with Na^+-containing buffer at room temperature for 1 to 3 h. If not stated otherwise, 10 μl of vesicles (0.1 mg protein) preloaded with Na^+ was diluted into 2 ml of Na^+-containing ($Na_i^+ = Na_o^+$) or Na^+-free buffer ($Na_i^+ > Na_o^+$) which, in addition, comprised 6 μM acridine orange. The measurement of fluorescence was started 5 s after the addition of vesicles.

The fluorescence was continuously recorded at room temperature in a Ratio Farrand Optical Co. Inc. fluorophotometer (excitation filter, 490 nm; emission filter, 511 nm). Initial rates of fluorescence quenching were estimated by drawing the tangent to the initial portion of the fluorescence recording and were expressed as fluorescence change per minute (ΔF/min). The initial rates in the presence of a Na^+ gradient were corrected for fluorescence changes observed in controls ($Na_i^+ = Na_o^+$). When present, valinomycin and monensin were added from ethanol stocks in a final concentration of 2.5 μM and 5 μM, respectively. Controls contained an equivalent amount (5 μl) of ethanol.

Kinetics of Na^+-H^+ exchange was determined from the rate of dissipation of a preset ΔpH by an inwardly directed Na^+ gradient. For this purpose, 10 μl of vesicles (0.1 mg protein), preloaded with K^+-buffer at room temperature for 2 h, was diluted into 2 ml TMA^+-buffer which contained 6 μM acridine orange and 2.5 μM valinomycin. This experimental set up led to the formation of transmembrane ΔpH [23–25]. When indicated (see legend to Fig. 3), 0.2 ml of concentrated solutions of either tetramethylammonium gluconate (control) or sodium gluconate were injected into the cuvette under continuous stirring and the rates of dissipation of ΔpH were recorded. The osmolarity of the injected solutions containing various Na^+ concentrations was kept constant by adding an appropriate amount of concentrated solution of tetramethylammonium gluconate. The initial rates of ΔpH dissipation recorded in the presence of Na^+ gradients were subtracted for the rates observed in controls and were expressed as fluorescence change per 5 s (ΔF/5 s). When required, amiloride was added from water stock to a final concentration of 0.5 mM. The fluorescence measurements in kinetic experiments were made in a Shimadzu RF 510 spectrophotofluorometer equipped with a recorded (excitation, 493 nm; emission, 525 nm).

The uptake of labeled D-glucose in isolated brush-border membrane vesicles was measured by the rapid filtration technique. The details of the experiment are given in the legend of Table IV.

The data are shown either from single experiments (recordings) representative for more repetitions or as means $\pm$ S.E. The differences between the corresponding data were analysed by Student's *t*-test and were considered significant at $P < 0.05$.

Chemicals. Estradiol-17β-diproprionate and testosterone propionate were obtained from Prolek (Belgrade, Yugoslavia). Acridine orange was

from Eastman Kodak (Rochester, NY, U.S.A). Valinomycin was from Calbiochem. (San Diego, CA, U.S.A.). Amiloride was a gift from Merck, Sharp and Dohme Research Laboratories (West Point, P.A., U.S.A.). Other chemicals were of analytical grade and were obtained commercially.

Results

Isolation of brush-border membrane vesicles

Table I summarizes the enrichment factors of the marker enzyme activities for various cellular structures in our preparations of rat renal brush-border membrane vesicles. The specific activity of leucine arylamidase, a marker for luminal membranes, was enriched about 12-fold. The enrichment of the marker enzyme activities for basolateral membranes, endoplasmic reticulum, mitochondria and red blood cells was less than 1.0 indicating a negligible contamination of luminal membrane preparations with the respective (intra)cellular membranes. Acid phosphatase activity, a lysosomal marker, however, was enriched about 3-fold. The pattern of enzyme enrichments, as shown here, is similar to that described in the original high-speed centrifugation method for isolation of brush-border membrane vesicles from the same source [17]. Furthermore, the yield of protein ($2.8 \pm 0.21\%$, $n = 7$) and of luminal membranes (leucine arylamidase activity, $41.4 \pm 5.4\%$, $n = 5$) in our preparations is also comparable to that described by Biber et al. [17]. By using the same method we have isolated the renal cortical luminal membranes from a single mouse with an efficiency similar to that in the rat (data not shown). However, to obtain more material for the fluorescence measurements we pooled the renal cortical tissue from 3 to 4 mice.

TABLE I

ENRICHMENT FACTORS OF THE MARKER ENZYME ACTIVITIES IN THE RAT RENAL BRUSH-BORDER MEMBRANE FRACTION

Shown are means ± S.E. of the number of experiments indicated by n.

Enzyme (specific marker for)	n	Enrichment factor
Leucine arylamidase (brush-border membrane)	42	11.8 ± 0.66
$(Na^+ + K^+)$-ATPase (basolateral membrane)	24	0.5 ± 0.11
KCN-resistant NADH oxidoreductase (endoplasmic reticulum)	8	0.6 ± 0.2
Succinate-cytochrome *c* oxidoreductase (mitochondria)	8	0.1 ± 0.05
Acid phosphatase (lysosomes)	8	3.0 ± 0.4
Acetyl cholinesterase (red blood cells)	8	0.9 ± 0.07

Validation of the measurement of Na^+-H^+ exchange in isolated brush-border membrane vesicles in a ΔpH developing method

Brush-border membranes isolated by a low-speed centrifugation are vesiculated and retain their transport properties. When vesicles were loaded with Na^+-buffer at room temperature for 2 h and diluted into acridine orange-containing Na^+-free buffer, a time-dependent drop of fluorescence was observed indicating the presence of Na^+-H^+ exchange in the membranes (Fig. 1A, $Na_i^+ > Na_o^+$). No significant quenching was recorded in the absence of a Na^+ gradient ($Na_i^+ = Na_o^+$). Both the final quenching (%) and the initial rates of quenching (ΔF/min) were dependent on amount of protein in the assay. However, only the initial rates exhibited a linear dependence in the range of 0.05 to 0.15 mg protein per assay (Fig. 1B). Therefore, the initial rates of fluorescence quenching were considered as the measure of Na^+-H^+ exchange. In all forthcoming experiments, the vesicle suspensions were adjusted to a protein concentration of 10 mg/ml. The fluorescence measurements were performed with the same amount of vesicles (10 μl, 0.1 mg protein). This amount of protein gave fluorescence changes which were within the linear range in Fig. 1B.

In order to exclude that the preloading time affects the rates of Na^+-H^+ exchange in these experiments we tested the initial rates of fluorescence quenching after rat renal brush-border membrane vesicles had been preincubated in a Na^+-buffer at room temperature for 1, 2 and 3 h. The observed initial rates were $16.8 \pm 1.65\ \Delta F$/min ($n = 5$), $16.3 \pm 1.40\ \Delta F$/min ($n = 5$) and $15.9 \pm 1.96\ \Delta F$/min ($n = 4$), respectively, indicating that complete loading of vesicles with Na^+ was accomplished already after 1 h of incubation. In further experiments the vesicles were loaded with Na^+-

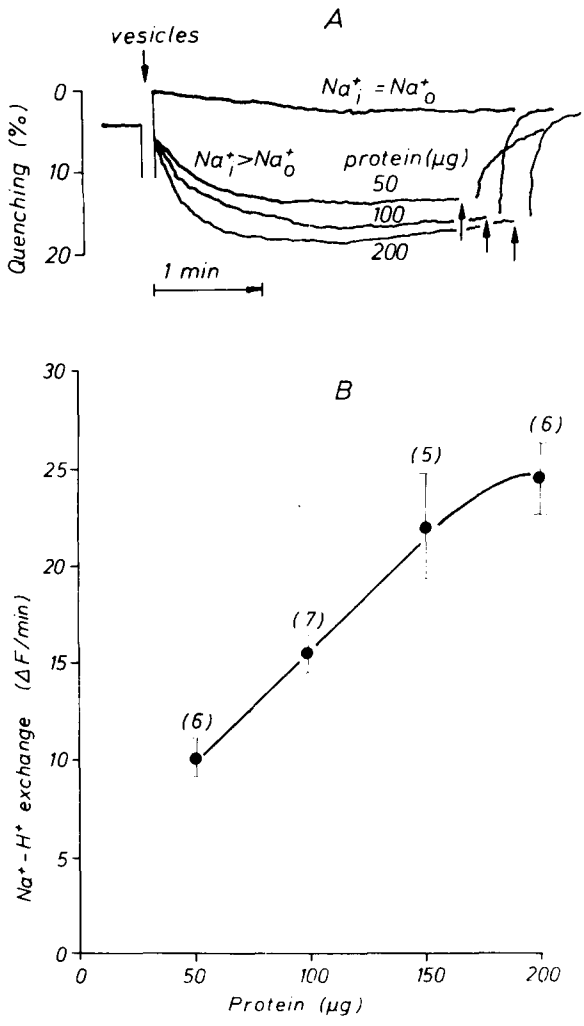

Fig. 1. Na^+-H^+ exchange in rat renal brush-border membrane vesicles demonstrated by a ΔpH developing method. (A) Na^+ gradient-driven quenching of acridine orange fluorescence and its dependence on protein concentration. Vesicles (10 to 20 μl containing the indicated amount of protein) were preloaded with Na^+-buffer and diluted into the same ($Na_i^+ = Na_o^+$) or TMA^+-buffer ($Na_i^+ > Na_o^+$). At the indicated time (↑) Na^+ was added to dissipate ΔpH. (B) Initial rates of acridine orange fluorescence quenching as a function of vesicle protein concentration. Shown are means ± S.E. for the number of experiments indicated in the brackets.

containing buffers at room temperature for 2 h.

Recent reports have described that Na^+-H^+ exchange can be due to the electroneutral Na^+-H^+ exchanger and to electrically coupled Na^+ and H^+ movements through parallel conductive pathways [23,26]. Moreover, it has been shown that the electrically coupled Na^+-H^+ exchange depends on membrane preparation, being significant (up to 55% of the total exchange) in membranes prepared by the Ca-precipitation method and negligible in membranes obtained with the Mg/EGTA-precipitation method [26]. Therefore, we have tested the contributions of electroneutral and electrically coupled Na^+-H^+ exchange in our preparations of rat renal luminal membranes. For this purpose, equimolar concentrations of K^+ were present on both sides of the vesicle membrane. In the presence of the K^+ ionophore valinomycin, an inside negative Na^+ diffusion potential as a possible driving force for H^+ uptake is abolished and only an electroneutral Na^+-H^+ exchange is recorded [23,26]. In four experiments the rates of Na^+-H^+ exchange in ethanol- and valinomycin-containing buffers were similar ($16.9 \pm 1.03\ \Delta F$/min and $17.0 \pm 1.79\ \Delta F$/min, respectively) indicating the absence of significant electrically coupled Na^+ and H^+ movements. This finding is in complete agreement with previous observations obtained with rat renal luminal membrane vesicles isolated by Mg/EGTA-precipitation at high-speed centrifugations [26]. As our membranes exhibited only electroneutral Na^+-H^+ exchange, further studies were made in the absence of valinomycin.

Effect of sex hormones on Na^+-H^+ exchange in rat and mouse renal brush-border membrane vesicles

Fig. 2 demonstrates the fluorescence quenching curves due to Na^+-H^+ exchange in brush-border membrane vesicles from the male rat and the mouse. The final quenchings ($Na_i^+ > Na_o^+$) were similar. The initial rates, however, in rat luminal membrane vesicles were twice as high as in mouse luminal membrane vesicles (Fig. 2, Table II). The observed differences can not be due to the differences in membrane preparations since the enrichment factors for the luminal membrane marker enzyme, leucine arylamidase, were similar in rat and mouse brush-border membranes (Table II). The enrichment in ($Na^+ + K^+$)-ATPase activity was also similar in both membrane preparations and varied between 0.1 and 0.6 (data not shown). Furthermore, by using an artificial Na^+-H^+ exchanger, monensin, we have tested the possibility that the degree of vesiculation, the buffering

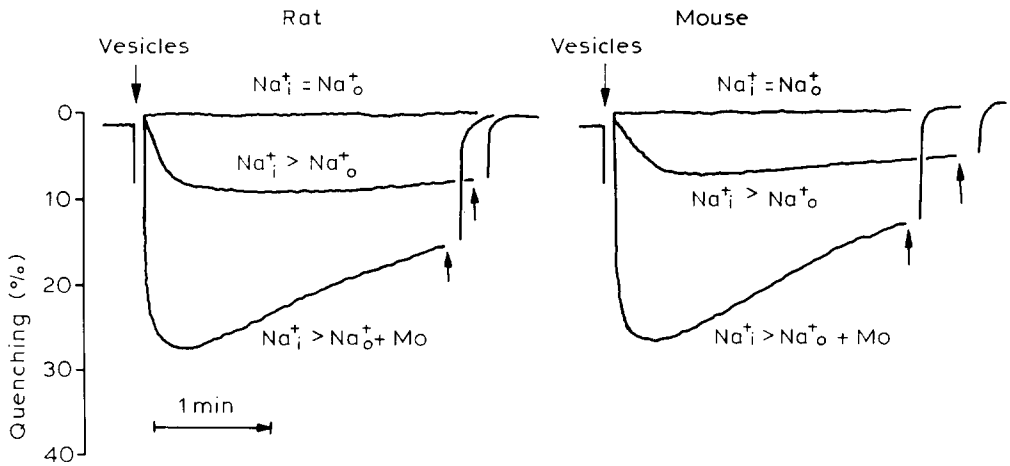

Fig. 2. Demonstration of Na^+-H^+ exchange in rat and mouse renal brush-border membrane vesicles. Vesicles (10 μl, 0.1 mg protein), preloaded with Na^+-buffer, were diluted into the same ($Na^+_i = Na^+_o$) or TMA^+-buffer which in addition contained either ethanol ($Na^+_i > Na^+_o$) or monensin ($Na^+_i > Na^+_o$ + Mo).

capacity, or the preloading with Na^+ were different in these two membrane preparations. A lower degree of vesiculation or buffering capacity or/and insufficient preloading with Na^+ should lead to a smaller fluorescence quenching in the presence of monensin. However, Fig. 2. indicates that all three possibilities can be excluded since in the presence of monensin the similar fluorescence changes were

TABLE II

LEUCINE ARYLAMIDASE ACTIVITY (LAP) AND Na^+-H^+ EXCHANGE IN RAT AND MOUSE RENAL BRUSH-BORDER MEMBRANE VESICLES; SEX DIFFERENCES AND EFFECT OF GONADAL HORMONES

Shown are the means ± S.E. for the number of experiments indicated in the brackets. Statistically significant differences ($P < 0.05$): [a] in comparison with oil-treated castrated females; [b] in comparison with male mouse; [c] in comparison with oil-treated castrated males.

Animals, sex and treatment	LAP (μmol·min^{-1}·(mg protein)$^{-1}$)			Na^+-H^+ exchange (ΔF·min^{-1})
	Homogenate	Vesicles	Enrichment factor	
Rat				
Males (12)	23.3 ± 2.26	279.7 ± 19.8	13.2 ± 1.01	16.3 ± 0.88
Females (14)	27.2 ± 2.58	281.2 ± 25.7	11.2 ± 0.91	15.8 ± 0.63
Castrated females				
+ oil (6)	20.2 ± 2.26	213.3 ± 14.9	11.0 ± 1.15	13.5 ± 1.00
+ estradiol (6)	27.9 ± 1.86 [a]	178.0 ± 7.9	6.6 ± 0.47 [a]	15.0 ± 0.68
+ testosterone (3)	25.7 ± 0.85	355.6 ± 65.3 [a]	10.4 ± 2.05	12.9 ± 0.71
Mouse				
Males (9)	14.4 ± 1.23	148.9 ± 14.9	10.8 ± 1.19	8.3 ± 0.76
Females (9)	18.9 ± 1.02 [b]	149.1 ± 9.7	7.9 ± 0.37 [b]	5.5 ± 0.51 [b]
Castrated males				
+ oil (10)	22.8 ± 2.66	210.2 ± 33.1	9.8 ± 1.33	5.4 ± 1.02
+ estradiol (3)	39.6 ± 6.78 [c]	286.5 ± 16.9	7.7 ± 1.27	4.8 ± 0.58
+ testosterone (10)	26.8 ± 4.70	316.9 ± 57.5	11.8 ± 3.00	11.1 ± 1.16 [c]

observed in membrane preparations from both sources. Thus, we conclude that species differences exist in the activity of Na^+-H^+ exchanger in renal brush-border membranes.

Na^+-H^+ exchange was the same in male and female rats. Moreover, neither ovariectomy itself nor treatment of ovariectomized rats with estradiol or testosterone had any significant effect on renal Na^+-H^+ exchange (Table II). The dosages and duration of treatment with hormones were the same as those used previously to study the response of alcohol dehydrogenase activity in the rat kidney cortex [22]. Rat renal alcohol dehydrogenase is inducible by estradiol but not by testosterone [22]. Such an effect of gonadal hormones on renal alcohol dehydrogenase was confirmed also in the present experiments; estradiol, but not testosterone, increased the activity of rat renal cortical alcohol dehydrogenase by 5-fold (data not shown). However, neither estradiol nor testosterone changed the renal mass in rats within the period of treatment (data not shown).

Contrary to the observations in the rat, sex differences in Na^+-H^+ exchange exist in mouse renal brush-border membranes, the activities in males being by 40% higher than in females (Table II). Somewhat smaller enrichment factors for leucine arylamidase in brush-border membranes from female kidneys may suggest a difference in membrane preparations as the cause of the observed sex differences in Na^+-H^+ exchange. However, the specific activity of leucine arylamidase was identical in isolated brush-border membranes from both sexes and no strict correlation can be observed between Na^+-H^+ exchange and enrichment factors in other experiments (Table II). The enrichment in $(Na^+ + K^+)$-ATPase activity was below 0.5 and similar in both membrane preparations (data not shown). The fluorescence quenching in the presence of monensin was also similar in renal brush-border membrane vesicles from male ($30.2 \pm 1.77\%$, $n = 4$) and female mice ($32.2 \pm 1.64\%$, $n = 3$) indicating a similar degree of vesiculation, buffering capacity and loading with Na^+ in both membrane preparations.

To investigate the cause of the observed sex differences, e.g. inhibition by estrogens or stimulation by androgens, male mice were castrated and treated with oil (controls), estradiol, or testosterone. The dosages of hormones and duration of treatment were the same as those used to study the induction of renal growth and alcohol dehydrogenase activity in the mouse [27,28]. In agreement with previously-published data [27,28] we also found an increase in kidney mass (by 55%) and alcohol dehydrogenase activity (by 13-fold) in testosterone-treated mice in comparison with oil-treated controls. As opposed to rats, estradiol had no effect on either of these parameters in mice (data not shown).

As shown in Table II, oil-treated castrated male mice had the same Na^+-H^+ exchange as intact female mice. Treatment of castrated animals with estradiol had no effect, but treatment with testosterone led to an increase in renal Na^+-H^+ exchange by more than 100% in comparison with controls. Again, neither the difference in enrichment factors for leucine arylamidase (Table II) or $(Na^+ + K^+)$-ATPase (data not shown) nor the fluorescence quenching due to monensin-mediated Na^+-H^+ exchange ($33.4 \pm 1.10\%$, $n = 4$ and $34.0 \pm 3.74\%$, $n = 5$ in controls and testosterone-treated mice, respectively) can account for the observed androgen effect. Therefore, these experiments indicate that the observed sex differences in mouse renal Na^+-H^+ exchange are caused by the stimulatory effect of androgen hormones.

Similar findings as observed above with the ΔpH developing method were also obtained in experiments in which the dissipation of a preset ΔpH was measured in response to inwardly directed Na^+ gradients. Here, K^+-loaded vesicles were diluted into valinomycin-containing K^+-free buffer which resulted in the development of a transmembrane ΔpH as described previously [23–25]. As shown for rat renal brush-border membranes vesicles (Fig. 3), a K^+ gradient-driven intravesicular acidification led to a final fluorescence quenching between 40% and 45%. Similar quenchings were also obtained with other membrane preparations (not shown). Addition of Na^+ to the cuvette after ΔpH had been fully developed led to an increased rate of ΔpH dissipation and was thus a measure of Na^+-H^+ exchange in the membranes. The recorded exchange is assumed to be electroneutral as both K^+ and valinomycin were already present in the measuring system. The rate of dissipation of ΔpH increased with increas-

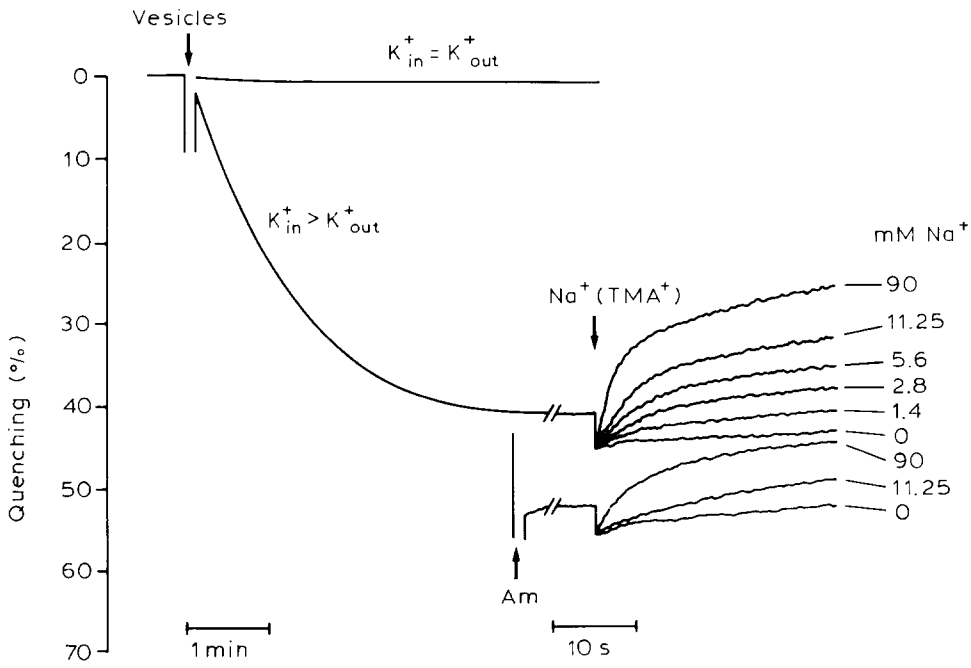

Fig. 3. Na^+-H^+ exchange in rat renal brush-border membranes demonstrated by a Na^+ gradient-driven dissipation of a preset ΔpH. Vesicles were preloaded with K^+-buffer and diluted into the same ($K^+_{in} = K^+_{out}$) or TMA^+-buffer ($K^+_{in} > K^+_{out}$) which contained valinomycin. After ΔpH in the presence of a K^+ gradient had fully developed, the speed of the recording was increased and either TMA^+ or various concentrations of Na^+ were injected into the cuvette (↓) under continuous stirring. When present, amiloride was added 40 s prior to the injection of a cation.

ing extravesicular Na^+ concentrations. Furthermore, we have tested the effect of amiloride, an inhibitor of electroneutral Na^+-H^+ exchange, upon Na^+ gradient-mediated dissipations of ΔpH. In accordance to previous studies [26], 0.5 mM amiloride added to the fully developed ΔpH increased quenching by 10% but did not affect the rate of dissipation of ΔpH in absence of Na^+ (Fig. 3, lower curves, 0). On the contrary, the Na^+ gradient-dependent rates of ΔpH dissipation were strongly inhibited at 11.25 mM and 90 mM Na^+_{out} indicating a specific inhibition of electroneutral Na^+-H^+ exchange by amiloride. The inhibition was not complete probably because of the limited sensitivity of the exchanger to amiloride [26].

By this appraoch we measured the kinetics of Na^+-H^+ exchange and the efficiency of inhibition by amiloride in renal luminal membrane vesicles isolated from male rats and mice and oil- or testosterone-treated female mice. (The response of female mice to testosterone treatment is similar to that in castrated males with respect to both, alcohol dehydrogenase activity [28] and renal hypertrophy). As shown in Fig. 4 A and B, the initial rates of Na^+-H^+ exchange exhibited a hyperbolic dependence on Na^+ concentrations in vesicles from all experimental groups of animals. At 0.5 mM amiloride and 11.25 mM Na^+_{out} we observed nearly complete inhibition of the exchanger activity. Data in Table III indicate clear species differences in V_{max} of the exchanger, in membranes from male mice being by 57% smaller than in membranes from male rats. The maximal rates of Na^+-H^+ exchange in vesicles from female mice were by 27% smaller than those from the males indicating again sex differences. Testosterone treatment in female mice resulted in an enhancement of the exchanger activity by 150%. The observed differences in V_{max} can not be due to differences in membrane preparations as the enrichment factors for leucine arylamidase were similar in all occasions (Table III). These findings

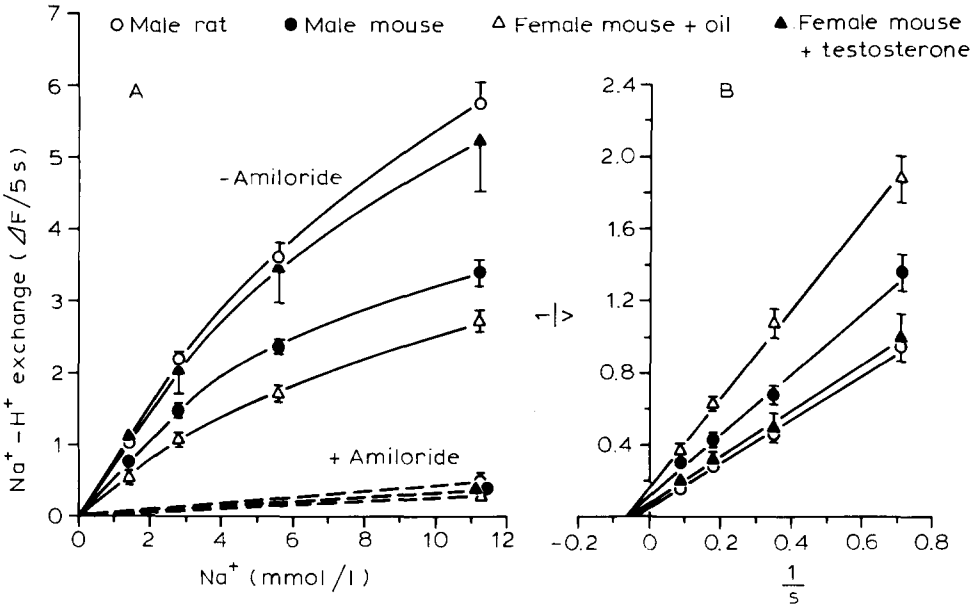

Fig. 4. (A) Na^+ concentration dependence of Na^+-H^+ exchange in renal brush-border membrane vesicles; influence of species, sex and treatment with testosterone. The experimental details are given in the legend of Fig. 3. (B) Lineweaver-Burk plot of the same data. Shown are the means ± S.E. of 5–8 experiments.

therefore are in complete agreement with the data obtained by the ΔpH developing method (Fig. 2, Table II). As also shown in Table III, Na^+-H^+ antiporter exhibits species differences also with respect to the affinity for Na^+, the K_m for Na^+ in membranes from mice being by 41% smaller than in membranes from rats. However, the K_m for Na^+ of the exchanger in mice was the same in both sexes and did not change after treatment of animals with testosterone.

TABLE III

ENRICHMENT FACTORS FOR LEUCINE ARYLAMIDASE ACTIVITY AND KINETIC CHARACTERISTICS OF Na^+-H^+ EXCHANGE IN RAT AND MOUSE RENAL BRUSH-BORDER MEMBRANES

Statistically significant differences ($P < 0.05$) were indicated by [a] (vs. male rats), [b] (vs. male mice) and [c] (vs. oil-treated female mice).

Animals and treatment	n	Enrichment factor	Na^+-H^+ exchange	
			V_{max} (ΔF/5 s)	K_m (mM Na^+)
Male rats	8	13.4 ± 1.15	21.0 ± 3.59	27.7 ± 6.10
Male mice	6	12.8 ± 0.51	9.1 ± 1.37 [a]	16.2 ± 3.05 [a]
Female mice				
+ oil	7	13.7 ± 1.05	6.6 ± 0.37 [b]	15.6 ± 0.87
+ testosterone	5	12.7 ± 1.39	16.4 ± 3.85 [c]	18.9 ± 3.09

Furthermore, we investigated species and hormonal effects on Na^+-dependent D-glucose uptake in brush-border membrane vesicles isolated from various groups of animals (Table IV). Na^+-dependent D-glucose uptake measured at 5 s (Table IV, Na^+-K^+) was by 20% smaller in membranes from male mice than in membranes from male rats. The uptake in female mice was only 40% of that observed in male mice indicating strong sex differences. After the female mice had been treated with testosterone, the intravesicular gucose uptake incrased by 200%. As also shown in Table IV, testosterone treatment resulted in 27% incrase of the equilibrium value for D-glucose uptake. This finding may indicate a slightly higher degree of vesiculation in these membrane preparations which, however, can not explain the much higher increase in Na^+-H^+ exchange activity and Na^+-dependent D-glucose uptake. Therefore we have to conclude that the testosterne effect in the mouse renal brush-border membranes is not restricted to the Na^+-H^+ exchanger, but also enhances D-glucose transport.

TABLE IV

EFFECT OF SPECIES AND SEX AND TESTOSTERONE TREATMENT ON D-GLUCOSE UPTAKE INTO RENAL BRUSH-BORDER MEMBRANE VESICLES

Vesicles were preloaded with 300 mM mannitol, 150 mM potassium gluconate, 5 mM Hepes-Tris (pH 7.0), and incubated for 5 s or 60 min (equilibrium) in buffers containing 100 μM labeled D-glucose, 300 mM mannitol, 150 mM NaCl (Na^+) or KCl (K^+), 5 mM Hepes-Tris (pH 7.0). Shown are the means ± S.E. of D-glucose uptake (pmol/mg protein) from 4–8 determinations (one experiment with vesicles pooled from 5–8 different preparations). Statistically significant differences ($P < 0.05$) were indicated by [a] (vs. male rats), [b] (vs. male mice) and [c] (vs. oil-treated female mice).

Animals and treatment	D-Glucose uptake			
	5 s uptake			Equilibrium
	Na^+	K^+	$Na^+ - K^+$	
Male rats	266.5 ± 7.48	93.3 ± 12.38	173.2	180.0 ± 11.74
Male mice	276.0 ± 9.27	139.5 ± 10.25 [a]	136.5	213.6 ± 10.20 [a]
Female mice				
+ oil	196.3 ± 4.28 [b]	141.1 ± 3.71	55.2	217.5 ± 7.86
+ testosterone	319.5 ± 11.98 [c]	152.1 ± 16.37	167.4	270.9 ± 22.74 [c]

Discussion

By using the fluorescence of acridine orange we studied sex differences and the effect of gonadal hormones on Na^+-H^+ exchange in brush-border membrane vesicles isolated by a modified Mg/EGTA-precipitation method from rat and mouse renal cortex.

The data presented in this paper show that brush-border membranes can be isolated from renal cortex at low gravitational forces (not more than $8000 \times g$). Such conditions can be achieved by using conventional table top centrifuges. The luminal membrane vesicles, thus obtained, possess enzymatic characteristics similar to those in membranes isolated by either free-flow electrophoresis [29] or high-speed centrifugations [17,30, 31]. The membrane vesicles exhibit a high rate of electroneutral Na^+-H^+ exchange and no significant electrically-coupled transmembrane movement of Na^+ and H^+ as was found for membrane vesicles isolated by the original high-speed centrifugation method [26]. Therefore, an easy and efficient method of isolation of renal luminal membrane vesicles is available which requires no expensive equipment.

Na^+-H^+ exchange in isolated renal luminal membranes is smaller in the mouse than in the rat thus exhibiting species differences. Although the rates of Na^+-H^+ exchange have been studied in a variety of physiological and pathophysiological conditions in rat and rabbit [2–11,26], species differences have not been described to our knowledge. The species differences demonstrated in this work may influence the interpretation and comparison of data on Na^+-H^+ exchange in renal brush-border membrane vesicles from various animals. Moreover, such a comparison can be further complicated by the effects of sex hormones on the activity of renal luminal Na^+-H^+ exchange. As described in this work, androgen hormones are strong stimulators of the renal Na^+-H^+ exchanger in mice, but not in rats. Thus, besides glucocorticoids and thyroid hormones, which are potent activators of renal Na^+-H^+ exchange in the rat [7–9], androgens possess similar stimulating efficiency in the mouse kidney. The stimulation by testosterone in the mouse kidney is restricted to the V_{max} of the exchanger without changing K_m for Na^+. Further studies should reveal whether testosterone treatment increases the number of transporting units in the membrane by affecting the rate of synthesis and/or degradation of the exchanger. Alternatively, testosterone could increase the conversion of an inactive form of the Na^+-H^+ antiporter into an active form or change the turnover rate of prexisting antiporters.

The modulations in activity of renal Na^+-H^+ exchanger are usually connected with changes in renal acid secretion and bicarbonate reabsorption.

For example, metabolic acidosis, hyperglucocorticoidism and chronic renal failure are associated with increased tubular H^+ secretion [33,34] and Na^+-H^+ exchanger activity [2,3,7,8]. On the contrary, parathyroid hormone inhibits both HCO_3^- reabsorption in isolated proximal tubule [35] and Na^+-H^+ exchange in renal brush-border membrane vesicles [11] from the rabbit. The enhanced activity of Na^+-H^+ exchanger in renal brush-border membrane vesicles from testosterone-treated mice should lead to an increased proton secretion. However, although testosterone elicits a number of receptor-mediated intracellular effects in mouse kidney that result in a significant organ growth [36], a possible effect of this hormone on H^+ secretion and HCO_3^- reabsorption has not been studied.

In accordance with recent findings of testosterone-induced stimulation of endocytosis, amino acid, and hexose transport in mouse kidney cortex slices [13,14] we found an enhanced Na^+-dependent uptake of D-glucose in renal luminal membranes isolated from testosterone-treated mice. These effects could be closely related to ornithine decarboxylase activity and intracellular concentration of polyamines which both strongly increase in the mouse kidney after testosterone treatment [13,14]. As measured in vitro, polyamines were found to stimulate Na^+-dependent D-glucose transport in isolated mouse renal brush-border membrane vesicles [16]. The same study, however, showed an inhibition of amiloride-sensitive Na^+-H^+ exchange by polyamines. This is in conflict with our findings that testosterone, administered in vivo, enhances Na^+-H^+ exchange in the mouse kidney. However, differences between hormonal effects on the membrane in vivo and in vitro can be substantial because of the possible loss of regulatory mechanisms in isolated membranes, as has already been mentioned [23].

Previous studies support the view that polyamines serve as intracellular signals to enhance free cytosolic Ca^{2+} concentration by increased Ca^{2+} influx across the plasma membrane and release from mitochondria and other organelles [15]. The increased cytosolic Ca^{2+} triggers the increase in endocytosis, hexose, and amino acid transport in mouse renal cortex. One of these targets could also be the Na^+-H^+ exchanger. Indeed, Ca^{2+} mobilization has been suggested to control Na^+-H^+ antiporter activity in a variety of eukaryotic cells [41–43] via either Ca^{2+}-calmodulin-dependent reactions [42] or via Ca^{2+}-sensitive, phospholipid-dependent protein kinase C activity [43,44]. Clearly, further studies are necessary to reveal the interactions between polyamines, Ca^{2+} fluxes, activity of protein kinase C, and Na^+-H^+ exchange in mouse kidney following testosterone treatment in vivo.

As indicated in this work, treatment by testosterone resulted in a significant growth of kidney mass in the mouse, but not in the rat. The renotropic action of testosterone in the mouse could be mediated through changes in Na^+-H^+ exchange activity as previously described for various growth factors. Increased Na^+-H^+ exchange takes place in see urchin eggs after fertilisation [37], in mitogen-stimulated lymphocytes [38], in fibroblasts activated by growth factors [39] and in the remnant kidney after unilateral nephrectomy [4].

In conclusion, we described the species differences in rat and mouse renal Na^+-H^+ exchange. Furthermore, Na^+-H^+ exchange and Na^+-dependent D-glucose transport in mouse kidney exhibit the strong sex differences determined by the potent stimulatory action of androgen hormones. The findings indicate that not only various intracellular phenomena [36] but also an increased transport of ions and solutes across the luminal plasma membrane contribute to the well known trophic action of androgens in the mouse proximal tubule.

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