

# Salt loading induces redistribution of the plasmalemmal Na/K-ATPase in proximal tubule cells

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## Salt loading induces redistribution of the plasmalemmal Na/K-ATPase in proximal tubule cells.

**Background.** We have reported that digitalis-like substances (cardiotonic steroids), including marinobufagenin (MBG), induce endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells. The current report addresses the potential relevance of plasmalemmal Na/K-ATPase redistribution to in vivo salt handling.

**Methods.** Male Sprague-Dawley rats were given 1 week of a high salt (4.0% NaCl) or normal salt (0.4% NaCl) diet. Urinary sodium excretion, as well as MBG excretion, was monitored, and proximal tubules were isolated using a Percoll gradient method. Tubular  $^{86}\text{Rb}$  uptake, Na/K-ATPase enzymatic activity, and Na/K-ATPase  $\alpha 1$  subunit density were determined.

**Results.** The high salt diet increased urinary sodium ( $17.8 \pm 1.8$  vs.  $2.5 \pm 0.3$  mEq/day,  $P < 0.01$ ) and MBG excretion ( $104 \pm 12$  vs.  $26 \pm 4$  pmol/day), and decreased proximal tubular  $^{86}\text{Rb}$  uptake ( $0.44 \pm 0.07$  vs.  $1.00 \pm 0.10$ ,  $P < 0.01$ ) and Na/K-ATPase enzymatic activity ( $5.1 \pm 1.1$  vs.  $9.9 \pm 1.6$   $\mu\text{mol}/\text{mg}$  pr/hr,  $P < 0.01$ ) relative to the normal diet. Proximal tubular Na/K-ATPase  $\alpha 1$  protein density was decreased in the plasmalemma fraction but increased in both early and late endosomes following the high salt diet. In rats fed a high salt diet, anti-MBG antibody caused a 60% reduction in urinary sodium excretion, substantial increases in proximal tubule  $^{86}\text{Rb}$  uptake, and Na/K-ATPase enzymatic activity, as well as significant decreases in the early and late endosomal Na/K-ATPase  $\alpha 1$  protein content.

**Conclusion.** These data suggest that redistribution of the proximal tubule Na/K-ATPase in response to endogenous cardiotonic steroids plays an important role in renal adaptation to salt loading.

Renal sodium handling involves a complex interplay of different hormonal systems and physical factors. Prox-

**Key words:** sodium, potassium, Na<sup>+</sup>/K<sup>+</sup>-ATPase, endocytosis, hypertension.

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imal tubular sodium reabsorption is felt to represent the bulk of sodium reabsorption, in some situations as high as 80% of all filtered sodium [1]. In the proximal tubule cell, the Na/K-ATPase resides at the basolateral surface, where it provides the force for the vectorial transport of sodium from the tubular lumen to the blood compartment [2]. The cellular distribution of the Na/K-ATPase is crucial for this function. During the 1960s, it was postulated that one or more inhibitors of the Na/K-ATPase similar to digitalis (digitalis-like substances, DLS) were present in the circulation, and were important in the regulation of proximal tubular sodium reabsorption and overall renal sodium handling [3]. Recent studies have demonstrated that substances that are structurally similar to ouabain of plant origin (OLC), as well as marinobufagenin (MBG), are involved in the regulation of blood pressure and natriuresis [4–8]. Moreover, in some cases, the increases in the concentrations of one of these cardiotonic steroids, MBG, correlates with increases in renal sodium excretion [9]. Cardiotonic steroids have also been found in hypothalamus and adrenal glands of animals [10]; one of these has been chemically identified as an isomer of ouabain [11], derived from plants. It was also found that the serum concentrations of cardiotonic steroids determined with immunoassays are increased in patients afflicted with some disease states associated with volume expansion [4, 6, 12–14]. Recent studies in rodents suggest that circulating levels of MBG have the strongest association with renal sodium excretion [5, 15]. In particular, MBG appears to have a much greater inhibitory effect on the rodent  $\alpha 1$  isoform of the Na/K-ATPase than ouabain, and it is the  $\alpha 1$  isoform that is almost exclusively expressed in rat kidney tissue [16]. Interestingly, administration of an anti-MBG antibody prior to an acute salt load dramatically attenuated the increases in urinary sodium excretion in Dahl, salt-sensitive rats [9].

We have previously attempted to address this topic using cell culture systems. Specifically, we have reported

that in LLC-PK1 cells (a model of proximal tubule cells), ouabain induces internalization of more than 50% of the plasmalemmal Na/K-ATPase over 12 hours at concentrations less than 1/20th of that necessary to acutely inhibit the plasmalemmal Na/K-ATPase by 50% (IC<sub>50</sub>). We have proposed that this mechanism might explain how cardiotoxic steroids circulating at concentrations of 10<sup>-9</sup> mol/L could substantially alter proximal tubule sodium handling in vivo [17]. This work comes on the background of extensive studies demonstrating that binding of cardiotoxic steroids to the Na/K-ATPase initiates a signal transduction cascade requiring sequential activation of Src, Ras, and MAPK [18–22]. Further studies with the LLC-PK1 cells demonstrate that ouabain-induced Na/K-ATPase endocytosis is a clathrin-dependent process that involves at least the beginning of this signaling cascade, specifically Src activation [23]. Interestingly, regulation of Na/K-ATPase activity by dopamine also appears to require endocytosis of the enzyme [24–30]. On this background, we hypothesized that redistribution of the sodium pump stimulated by circulating digitalis-like substances plays an important role in the renal adaptation to a high salt diet. To test this hypothesis, the following studies were performed.

## METHODS

### Animals

All animal experiments and procedures were performed in accordance with the guidelines set by the NIH, and were approved by the Internal Animal Care and Use Committee of Medical College of Ohio.

Male Sprague-Dawley rats (weights 300 g) were purchased from Charles River and maintained in our animal division with 12-hour dark/light cycle. After two days of adjustment to the new environment, the animals were divided into different dietary groups; one group received a standard rodent diet (0.4% NaCl), and the other group a high salt diet (4.0% NaCl). All rats had ad libitum access to the food and water. In some experiments, rats were transferred to metabolic cages (one rat/cage) after at least six days of the normal or high salt diet and maintained on the same diet, while urine samples were collected for 2 or 3 days. Urine samples were analyzed for volume, Na concentration, using atomic absorption spectroscopy, as well as marinobufogenin (MBG) concentration, using antiserum developed in our laboratory (MBG-P), and dissociation-enhanced lanthanide fluorimunoassay (DELFLIA) [5, 31]. In some animals, conscious blood pressure (BP) was measured by the tail cuff method [32] in conscious, restrained rats with equipment made by IITC, Inc. (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA, USA). Rats were acclimated to the test chamber and warmed to 30°C prior to BP measurement. At least three

consistent BP readings at a given daily session were obtained and averaged as that session's reading.

In some cases, animals were subjected to a high salt diet for 7 days and then placed in a metabolic cage. Both food consumption and urine volume were monitored. Baseline urine was collected for 48 hours, and then two doses of either anti-MBG (antiserum-V10) or nonimmune serum, 100 µL, were injected through the tail vein 24 hours apart. This dose was chosen because of its profound effect on urinary MBG excretion and urinary sodium excretion in Dahl salt-sensitive rats. Anti-MBG serum (or nonimmune serum) was dialyzed using Spectra/Por membrane (molecular weight cutoff: 12–14 kD) against Tris-buffered saline overnight at 4°C as previously reported [9]. Urine was collected during this time, and the rats were then sacrificed 24 hours after the second dose of antibody (or nonimmune serum). Anti-MBG antibody titers were determined using enzyme-linked immunosorbent assay (ELISA) plates coated with an MBG-thyroglobulin conjugate. The antigen-antibody complex was detected using a secondary Ab conjugated to alkaline phosphatase reactive to rabbit IgG (V-10 was raised in rabbits) and an ELISA amplification kit (Invitrogen, Carlsbad, CA, USA). In each rat sample measured, anti-MBG titers ranged between 1:10,000 and 1:100,000 in rats given V-10 (*N* = 10), whereas they were not detectable in the rats given nonimmune serum (*N* = 10). In some cases, renal cortical tubules were isolated (as described below) for further study.

### Isolation of renal cortical tubules

The method for isolation of renal cortical tubules segments was modified from Vinay et al [33]. All solutions used for isolation were equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 1 hour prior to use, and their pH was adjusted to 7.4. All procedures were carried out on ice except the digestion of tissues with collagenase. Rats were anesthetized with pentobarbital (50 mg/kg, IP) and, through a midline incision in the abdomen, the kidneys were excised, decapsulated, and cut into two halves. The renal cortex was dissected out, finely minced, and then transferred to a flask containing Dulbecco's modified Eagle's medium (DMEM) plus collagenase. The tissue was incubated at 37°C in shaking water bath with continuous oxygenation. After 15 minutes' digestion, the cortical tissues were mechanically disrupted by passing through an 18-gauge needle, and the supernatant containing crude tubule segments were removed from the flask. Fresh enzyme solution was added to the flask and incubated for another 15 minutes, and the tissues were passed through the needle as before, and the tubule suspension was harvested. This process was repeated two more times. By this procedure we were able to maximize the yield of cortical

tubules. The pooled cortical suspensions were washed three times with DMEM to remove residual collagenase.

### Separation of cortical tubules

The washed tubules were suspended in 30 mL of 42% Percoll solution (pH 7.4) made isotonic with DMEM. The tubular suspension in Percoll was centrifuged at 20,000 rpm for 30 minutes without braking. This centrifugation step separated the tubules into four or five major fractions. The diffuse upper band (F0) contained individual and broken cells and was discarded. The next two successive bands (F1 and F2) were enriched with distal tubules consisting of mostly intact shorter segments, as revealed by the microscopic examination. The bottom two bands (F3 and F4) were enriched with proximal tubules mostly of longer and broader segments. The F4 and F1 fractions were collected and washed three to four times with DMEM (to get rid of the Percoll), and were used immediately for measurement of the Na-pump activity. Our method of isolation and purification yielded a highly purified preparation of proximal (>95%) and distal tubules (>75%) determined with microscopy. The F4:F1 alkaline phosphatase and hexokinase activity ratios were  $2.4 \pm 0.4$  and  $0.2 \pm 0.1$  (mean  $\pm$  SD) for these enzymes expressed only in proximal and distal tubule epithelium, respectively. These data were quite similar to that reported by Vinay et al [33]. The viability of both F4 and F1 were >95% as determined by trypan blue exclusion.

### Measurement of Na-pump activity in cortical tubules

Measurement of ouabain-sensitive  $^{86}\text{Rb}$  uptake was used as an index of sodium pump activity. Ouabain-sensitive  $^{86}\text{Rb}$  uptake was measured in triplicate at 37°C using the tubule segments isolated as described above. The uptake of  $^{86}\text{Rb}$  by the tubules was carried out in DMEM containing 5.0  $\mu\text{mol/L}$  monensin, which was added to maximize the pump activity. The  $^{86}\text{Rb}$  uptake was determined in the presence and absence of 2.5 mmol/L ouabain to determine ouabain-sensitive  $^{86}\text{Rb}$  uptake as we have previously described [17, 34].

### Measurement of Na/K-ATPase activity in cortical tubules

The distal or proximal tubules were pelleted from the DMEM and washed three times with a hypotonic medium (25 mmol/L imidazole pH 7.4) to remove all the ions. The tubules were then made accessible to the substrates by subjecting them to four freeze/thaw cycles in the hypotonic medium. Na/K-ATPase activity of the permeabilized tubules was determined under  $V_{\text{max}}$  conditions by a radiochemical assay based on the measurement of phosphate released from ( $\gamma$ - $^{32}\text{P}$ ) ATP by the enzyme. The activity was measured in a total volume of 1.0 mL containing 50 mmol/L Tris-HCl pH 7.4, 4 mmol/L  $\text{MgCl}_2$ , 2 mmol/L

EGTA, 100 mmol/L NaCl, 10 mmol/L KCl, 5 mmol/L  $\text{NaN}_3$ , 3 mmol/L ( $\gamma$ - $^{32}\text{P}$ )-ATP, and 200 to 220  $\mu\text{g}$  of tubular protein, and in the presence and absence of 2.5 mmol/L ouabain. After a 30-minute preincubation of tubules with ouabain at 37°C, enzymatic reaction was initiated by the addition of ( $\gamma$ - $^{32}\text{P}$ )-ATP and terminated 10 minutes later by the addition of ice-cold 8% perchloric acid. Released inorganic ( $^{32}\text{P}$ )-phosphate was measured as described by Askari et al [35], and previously reported from our laboratory [17]. The difference between ATPase activity in the presence and absence of ouabain was considered as Na/K-ATPase activity. The ATPase activity was measured in triplicate, and the activity was expressed as  $\mu\text{mol}$  of phosphate released/mg of protein/hr.

### Determination of Na-pump expression in cortical tubules by Western blot

The tubules were washed three times with a hypotonic medium (25 mmol/L imidazole pH 7.4) and homogenized in the same medium. An aliquot of the homogenate was removed, and its protein content was determined [36]. The homogenates were solubilized in sample buffer (2% SDS, 5%  $\beta$ -mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, and 50 mmol/L Tris-HCl pH 7.0). The solubilized proteins were size fractionated on 10% polyacrylamide mini-gels under denaturing and reducing conditions as described by Laemmli [37]. The separated proteins were electrophoretically transferred to nitrocellulose membrane following the method of Towbin et al [38], and immunoblotted with anti- $\alpha$ 1 Na/K-ATPase mAb (Upstate, Waltham, MA, USA). The immunoreactive products were visualized with horseradish peroxidase-conjugated to goat antimouse IgG (Affinity Bioreagents, Inc., Golden, CO, USA) using an enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA). The images of the immunoreactive products captured on light-sensitive imaging films were quantified with a Molecular Analyst software program (Bio-Rad, Hercules, CA, USA).

The protein contents of cortical tubules were determined by bicinchoninic acid (BCA) assay [36] using bovine serum albumin as standard.

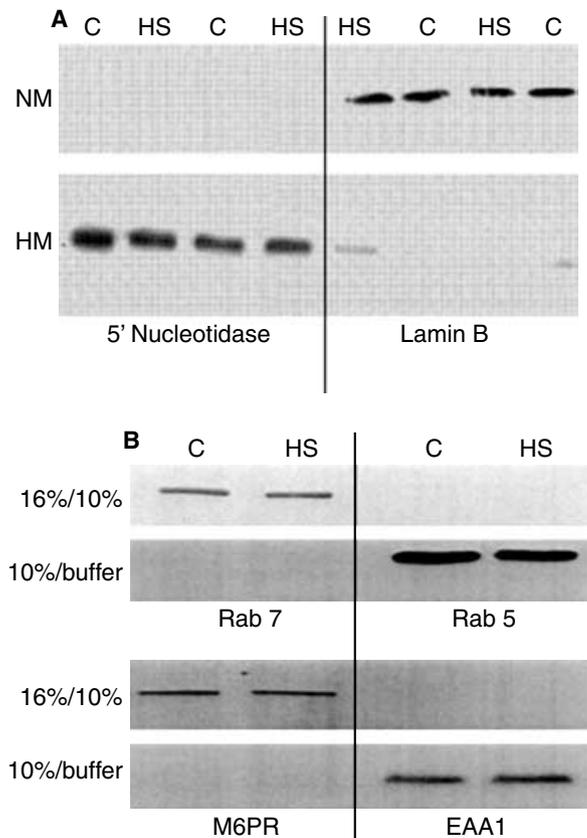
### Subcellular fractionation

Proximal tubules (10–15 mg) were collected with hypotonic buffer (10 mmol/L Tris-HCl, pH 7.2, 1 mmol/L PMSF, 10  $\mu\text{g/mL}$  of aprotinin, and 10  $\mu\text{g/mL}$  of leupeptin) on ice and allowed to sit on ice for 15 minutes. Cells were then separated into nuclear and heavy membrane compartments as we have previously reported [17] using the method of Akao et al [39]. The cell suspension was then passed through a syringe needle (26-gauge) 10 times, and then homogenized 15 times with a ball bearing cell homogenizer (H&Y Enterprise, Redwood

City, CA, USA). An equal volume of 0.5 mol/L sucrose buffer (in 10 mmol/L Tris-HCl, pH 7.2) was added to make the solution isotonic. The cell suspension was centrifuged at 750g at 4°C for 10 minutes to get the post-nuclear supernatant and the nuclear pellet. After three washes with isotonic buffer, the nuclear pellet was resuspended and passed through a 26-gauge needle several times, then centrifuged at 430,000g for 2 hours to pass through a 2M sucrose cushion. The post-nuclear supernatant was centrifuged at 17,000g at 4°C for 15 minutes to get the heavy membrane (plasmalemmal) pellet. The nuclear pellet showed very little contamination with plasmalemma, as assessed by the immunoblotting against markers to nuclear (Lamin B) and plasmalemma (5' nucleotidase, both antibodies obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalog numbers sc-6216 and sc-14684, respectively). Moreover, this minimal contamination was not affected by changing animals from a normal to high salt diet (Fig. 1A). All pellets were resuspended in ice-cold PBS with 1% NP-40 and 0.25% sodium deoxycholate, and stored at -80°C before further study.

### Preparation of endosomes

Endosomes were fractionated on a flotation gradient using the technique of Chibalin [40]. Briefly, proximal tubules (55 mg) were washed twice with ice-cold PBS-Ca-Mg (PBS containing 100 µmol/L CaCl<sub>2</sub> and 1 mmol/L MgCl<sub>2</sub>) and once with ice-cold PBS. These tubules were disrupted and centrifuged for 5 minutes at 4°C and 3000g. The cell pellet was resuspended in 3 mL of the homogenization buffer (250 mmol/L sucrose in 3 mmol/L imidazole, pH 7.4) and recentrifuged for 10 minutes at 4°C at 3000g. This pellet was then resuspended in 1.0 mL of homogenization buffer (with 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mmol/L PMSF, and 0.5 mmol/L EDTA), and gently homogenized on ice using a Dounce homogenizer (15–20 strokes). The homogenate was then centrifuged for 10 minutes at 4°C at 3000g. The supernatant, which contained 18 to 25 mg of protein, was then adjusted to 46% sucrose using a stock solution of 62% sucrose in 3 mmol/L imidazole (pH 7.4) and loaded at the bottom of a centrifuge tube, to which we sequentially added 16% sucrose (4 mL) in 3 mmol/L imidazole and 0.5 mmol/L EDTA in <sup>2</sup>H<sub>2</sub>O (Sigma-Aldrich, St. Louis, MO, USA), 10% sucrose in the same buffer (3 mL), and finally homogenization buffer (1 mL). The gradient was centrifuged for 60 minutes at 4°C and 130,000g in a Beckman SW 41Ti rotor (Beckman Coulter, Fullerton, CA, USA). Material obtained from the 16%/10% interface contained between 315 to 330 µg of protein. Material obtained from the 10%/buffer interface contained between 55 to 65 µg of protein. There was no difference in protein yield between rats fed either a high or normal salt diet.



**Fig. 1. (A) Representative Western blots against 5' nucleotidase and lamin B in nuclear membrane (NM) and heavy membrane (HM) isolated from proximal tubules of rats fed a normal salt (C) or high salt (HS) diet.** Each lane was loaded with 25 µg of protein. Note that contamination of the nuclear fraction by plasmalemma by immunoblotting against 5' nucleotidase is quite low. By developing film for a much longer period of time, we can estimate contamination of nuclear membrane fraction by heavy membrane fraction at less than 1:30 with no difference between normal and high salt diets. Conversely, we see contamination of heavy membrane fraction by nuclear fraction by immunoblotting against lamin B is also small, but is detectable on the Western blot shown. (B) Representative Western blots against Rab7, Rab5 (top two rows), and mannose 6-phosphate receptor (M6PR) and early endosomal antigen 1 (EAA1) (bottom two rows) in fractions obtained from the 16%/10% and 10%/buffer interfaces of proximal tubules isolated from rats fed normal (C) and high salt (HS) diets. For both Rab 5 and 7, each lane was loaded with 50 µg of protein. For M6PR and EAA1, lanes were loaded with 30 µg of protein. For M6PR, nonreducing conditions and a 7% gel were used. For other proteins, reducing conditions and 10% gels were used.

Materials from these interfaces were probed for Rab5, Rab7, mannose 6 phosphate receptor (M6PR), and early endosomal antigen 1 (EAA1) using Western blots performed as described above and antibodies obtained from Santa Cruz Biotechnology. Interestingly, we found that the sample harvested from the 16%/10% interface reacted positively with antibodies to Rab7 and M6PR (but not Rab5 or EAA1), whereas material harvested from the 10%/buffer interface reacted to Rab5 and EAA1 (but not Rab7 or M6PR) (Fig. 1B). Therefore, we subsequently refer to the material obtained from the 16%/10% and

10% buffer interfaces as late and early endosomes, respectively, consistent with that reported by Chibalin et al with rat proximal tubule cells [40], but different from that seen in our own earlier report on LLC-PK1 cells [23].

### Statistical analysis

Data obtained were compared using the unpaired or paired Student *t* test with Bonferroni's correction for multiple comparisons depending on the unpaired or paired nature of the data. Statistical analysis was performed using SPSS™ software (Chicago, IL, USA).

## RESULTS

### Effect of high salt diet on renal sodium handling

Rats were fed a normal diet (0.4% NaCl, *N* = 5) or high salt diet (4.0% NaCl, *N* = 5) for 7 to 10 days. Urine was collected for the last 24 hours, and the animals were sacrificed. At time of sacrifice, the groups did not differ with respect to body, heart, or kidney weight. Some additional animals were given a high salt (*N* = 10) or normal diet (*N* = 10) diet for 7 to 10 days, upon which conscious blood pressure was determined using the tail cuff method. Neither systolic (114 ± 5 vs. 107 ± 6 mm Hg), diastolic (79 ± 4 vs. 76 ± 5 mm Hg), nor mean blood pressures (91 ± 4 vs. 86 ± 6 mm Hg) were significantly different between rats fed a normal versus high salt diet, respectively. These data were consistent with the lack of effect of a high salt diet on blood pressure in Sprague-Dawley rats reported previously by members of our group [41].

Rats fed the high salt diet did demonstrate marked increases in urinary sodium (17.8 ± 1.9 vs. 2.5 ± 0.3 mEq/day, *P* < 0.01) and MBG (104 ± 12 vs. 25.6 ± 4.4 pmol/day, *P* < 0.01) excretion compared with rats maintained on a control diet (Figs. 2A and B).

### Effect of high salt diet on tubular Na/K-ATPase activity

Renal tubules were isolated from rats fed a normal or high salt diet, and studied for <sup>86</sup>Rb uptake, Na/K-ATPase activity, and Na/K-ATPase α1 protein density. The high salt diet did not cause any changes in <sup>86</sup>Rb uptake (1.13 ± 0.11 vs. 1.00 ± 0.08), and Na/K-ATPase α1 protein density (1.00 ± 0.06 vs. 1.00 ± 0.07) in the distal tubules (F1 fraction, both *P* = NS). In contrast, <sup>86</sup>Rb uptake and Na/K-ATPase activity in the proximal tubules (F4 fraction) were decreased by approximately 60% in rats fed a high compared with normal salt diet (Fig. 2C and D). However, proximal tubular Na/K-ATPase α1 protein density was not different between those rats fed a high and normal salt diet (Fig. 2E and F).

### Effect of high salt diet on distribution of Na/K-ATPase in proximal tubules

Because of this marked reduction in proximal tubule <sup>86</sup>Rb uptake and Na/K-ATPase enzymatic activity

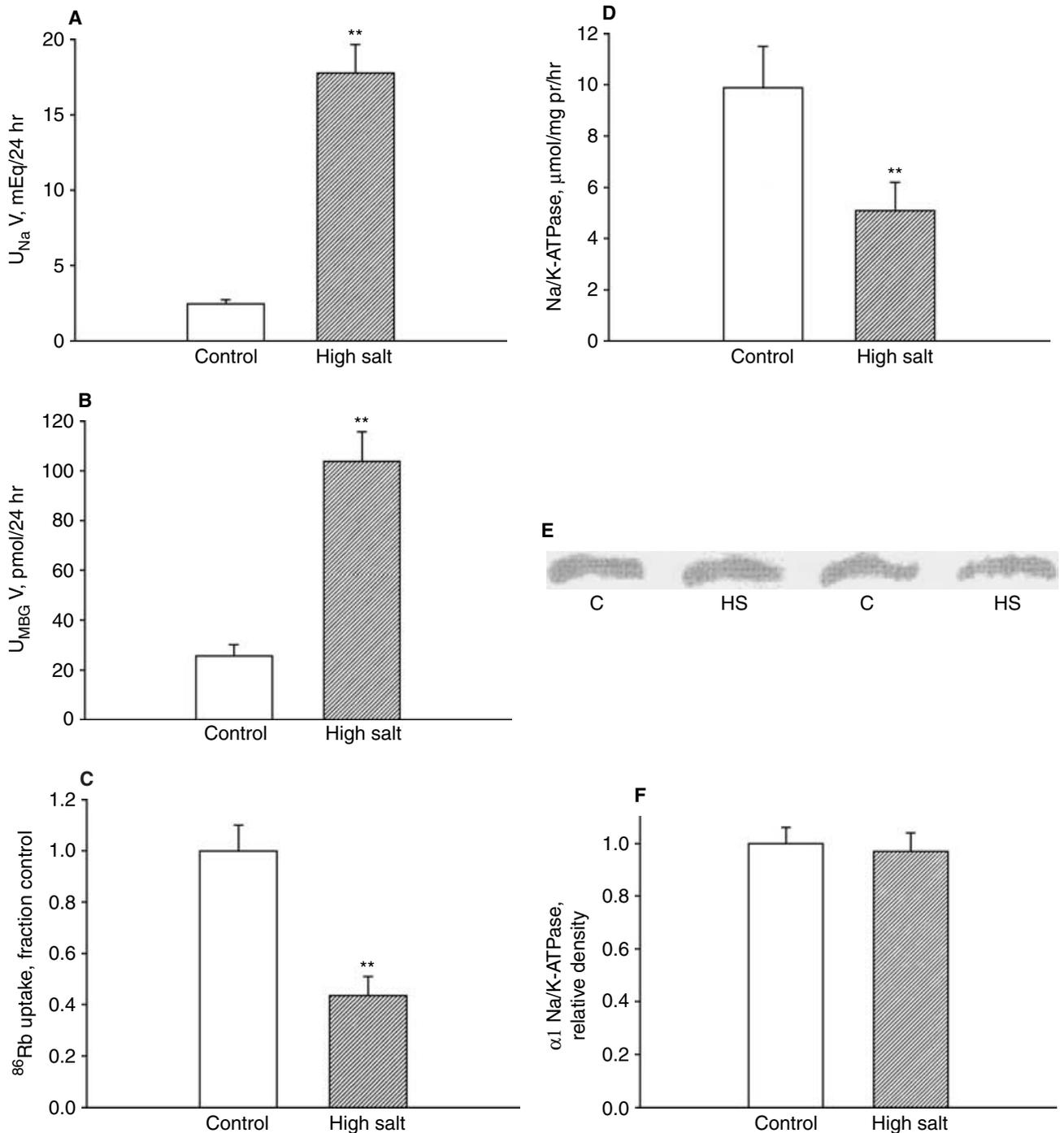
without an associated change in protein content, we performed the following studies to examine the cellular distribution of the Na/K-ATPase in these proximal tubule cells.

First, we examined the heavy membrane fraction from proximal tubules. When these proximal tubules were isolated from rats fed a high salt diet, this fraction showed a 50% reduction in Na/K-ATPase α1 subunit density compared with those isolated from rats fed a normal diet (Fig. 3).

We next examined if the endosomes of proximal tubules became enriched in Na/K-ATPase after the animals were exposed to the high salt diet for one week. Both early and late endosomes were substantially enriched in the α1 Na/K-ATPase in the proximal tubules isolated from rats fed a high salt versus control diet (Fig. 4).

### Effect of anti-MBG antibody on renal sodium handling and tubular Na/K-ATPase activity

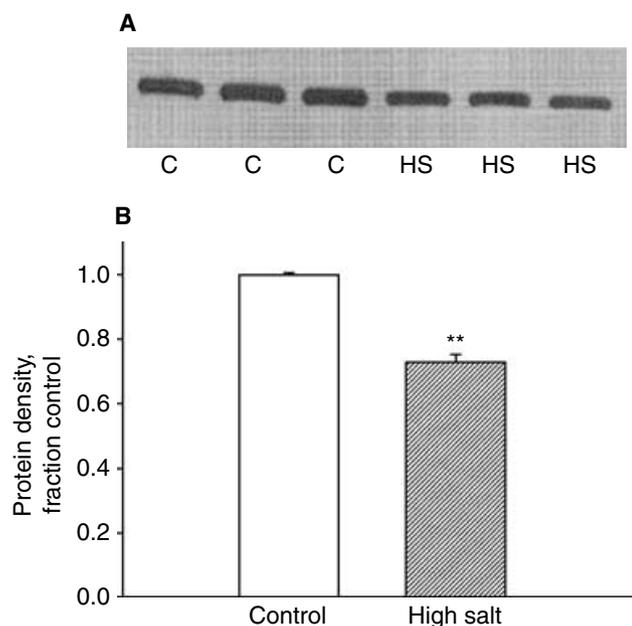
Following 7 to 10 days on the high salt diet, rats were placed into metabolic cages. The rats were treated with either 100 μL of anti-MBG antibody or nonimmune serum given intravenously 48 and 24 hours prior to sacrifice. Urine MBG excretion was calculated for the 24 hours prior to administration of antibody (or nonimmune serum) and for the 24 hours immediately following administration. Urine was also collected for measurement of Na excretion for 48 hours (two 24 hour collections) prior to administration of antibody (or nonimmune serum), as well as for the two 24 hour periods after administration. MBG excretion prior to administration (102 ± 8 pmol/24 hrs, *N* = 45 urine samples, 28 rats) was similar to that seen in other animals on the high salt diet (Fig. 2). Nonimmune serum did not significantly change MBG excretion at 24 (120 ± 10 pmol/24 hrs, *N* = 13) or 48 hours (95 ± 17 pmol/24 hrs, *N* = 9), whereas administration of the antibody resulted in a marked decrease in MBG excretion over this same time period (62 ± 6 pmol/24 hrs at 24 hrs, *N* = 14, *P* < 0.01 and 58 ± 14 pmol/24 hours at 48 hrs, *N* = 9, *P* < 0.05). Rats treated with the nonimmune serum also did not have significant changes in either their urinary sodium excretion (UNaV) or dietary sodium intake (Fig. 5). Moreover, proximal tubules isolated from these animals had <sup>86</sup>Rb uptake and Na/K-ATPase enzymatic activities indistinguishable from rats given the high salt diet alone (Fig. 6), as well as similar plasmalemmal (heavy membrane) and early and late endosomal content of the Na/K-ATPase α1 subunit (Fig. 7). However, rats treated with the anti-MBG antibody had a marked reduction in their UNaV without substantial change in dietary sodium intake (Fig. 5). Moreover, the proximal tubules from rats treated with the anti-MBG Ab demonstrated marked increases in proximal tubule <sup>86</sup>Rb uptake and Na/K-ATPase enzymatic



**Fig. 2.** Effect of high salt diet on urinary sodium excretion ( $U_{NaV}$ ) (A), urinary MBG excretion ( $U_{MBGV}$ ) (B), proximal tubular ouabain-sensitive  $^{86}Rb$  uptake (C) and Na/K-ATPase enzymatic activity (D), as well as Na/K-ATPase  $\alpha 1$  protein density (E and F). In (E), a representative Western blot is shown, whereas (F) shows quantitative protein data. Gels were loaded with 3  $\mu\text{g}$  of protein, which was within the linear range for detection for both the C and HS samples. Data from proximal tubules isolated from rats fed either a control or high salt diet. All data expressed as mean  $\pm$  SEM of 5 determinations in each group. \*\* $P < 0.01$  vs. control.

activity (Fig. 6), as well as increases in the plasmalemmal Na/K-ATPase  $\alpha 1$  subunit content, to levels seen in rats fed a control (0.4% sodium) diet (Fig. 7). We also noted that both the early and late endosomes isolated from the proximal tubules of rats treated with the anti-

MBG Ab showed substantial decreases in Na/K-ATPase  $\alpha 1$  subunit content compared with those treated with non-immune serum, but that the Na/K-ATPase  $\alpha 1$  subunit content did not return to values seen in the rats fed a control diet.



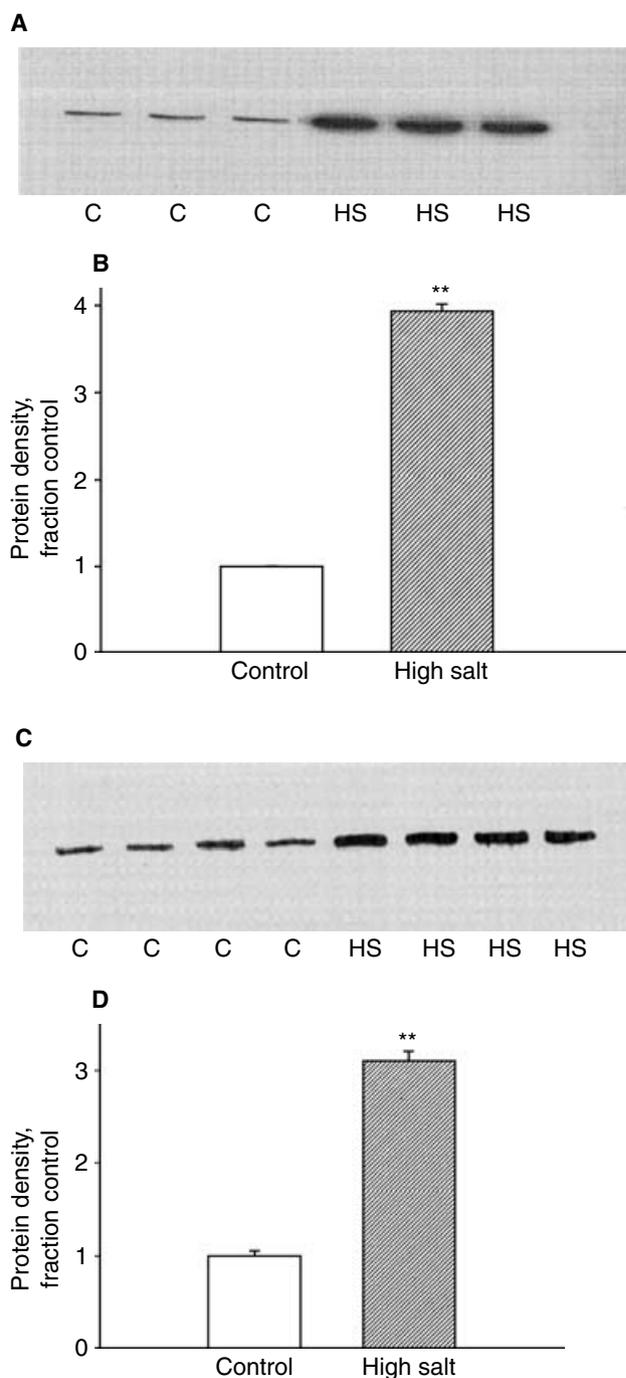
**Fig. 3. Effect of high salt (HS) diet on Na/K-ATPase  $\alpha$ 1 protein density determined in the plasmalemma fraction of proximal tubules compared with control (C).** (A) Shown is a representative Western blot, whereas (B) shows quantitative data. Gels were loaded with 3  $\mu$ g of protein, which was within the linear range for detection for both the C and HS samples. Data shown as mean  $\pm$  SEM of 10 determinations in each group. \*\* $P < 0.01$  vs. control.

## DISCUSSION

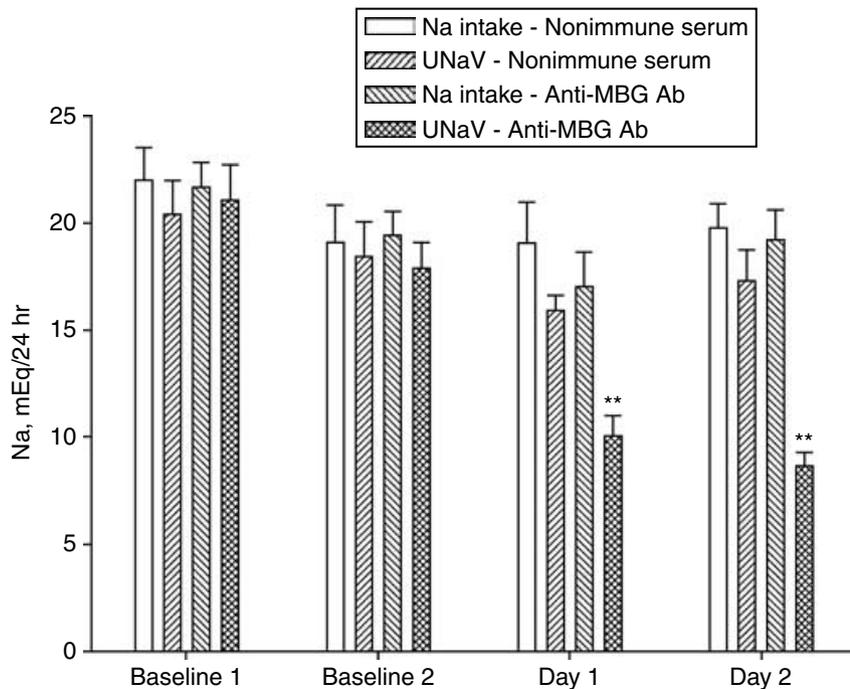
The role of circulating inhibitors of Na/K-ATPase in renal sodium homeostasis is still unclear despite decades of investigation [42]. Recently, it has been established that increases in the renal excretion and plasma concentration of cardiotonic steroids, such as MBG and OLC, accompany acute volume expansion in the Dahl salt-sensitive rat, and that administration of antibody to cardiotonic steroids blocks both the hypertension and the increases in urinary sodium excretion seen in this setting [5]. Based on data described in the current report, as well as data which we previously reported [17], we would suggest that cardiac steroid-induced endocytosis of the plasmalemmal Na/K-ATPase in proximal tubule cells may be an important mechanistic step linking the increases in cardiac steroid concentrations to the observed natriuresis.

In a recent report, we documented that ouabain and MBG induce marked endocytosis of the plasmalemmal sodium pump in LLC-PK1 cells. In fact, we observed that this endocytosis process substantially altered cellular  $^{86}\text{Rb}$  uptake and transcellular sodium transport [17]. Interestingly, we did not see these decreases in  $^{86}\text{Rb}$  uptake and Na/K-ATPase protein content in MDCK cells, which are generally used as a model of distal renal tubule epithelial cells [17].

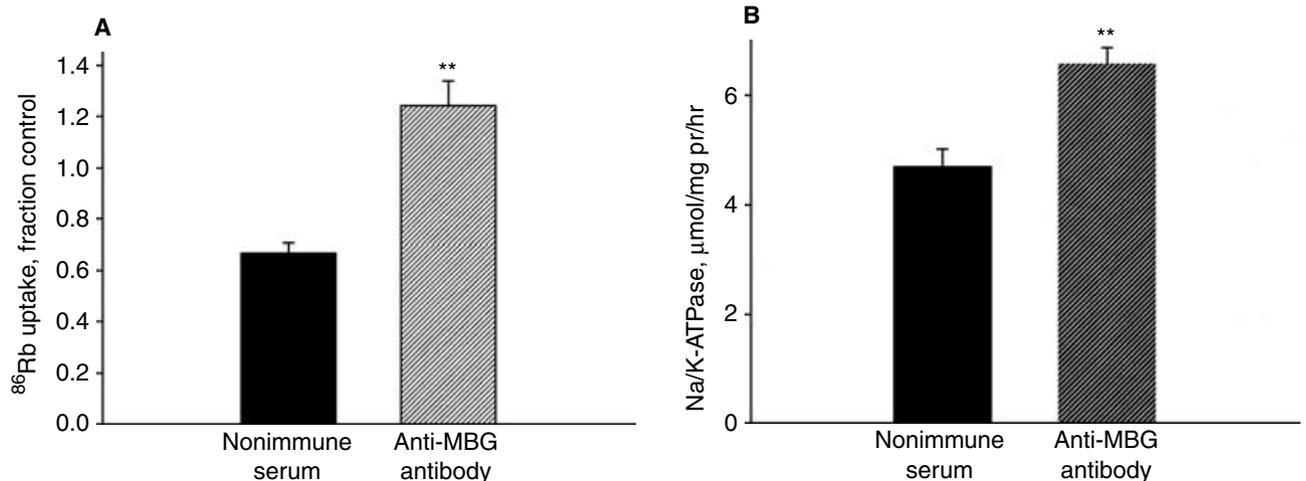
In the current paper, we documented that a high salt diet is associated with marked increases in the excretion



**Fig. 4. Effect of high salt (HS) diet on Na/K-ATPase  $\alpha$ 1 protein density determined in early and late endosomes isolated from proximal tubules compared with control (C).** (A) Shown is a representative Western blot obtained from early endosomes, whereas (B) shows quantitative data. (C) Shown is a representative Western blot obtained from late endosomes, whereas (D) shows quantitative data. Gels for early endosomes were loaded with 30  $\mu$ g of protein and late endosomes were loaded with 25  $\mu$ g of protein. Both of these values were within the linear range for detection for both C and HS samples. Data shown as mean  $\pm$  SEM of 6 determinations for the early endosomes in both the C and HS groups, and 12 determinations for the late endosomes for both the C and HS groups. \*\* $P < 0.01$  vs. control.



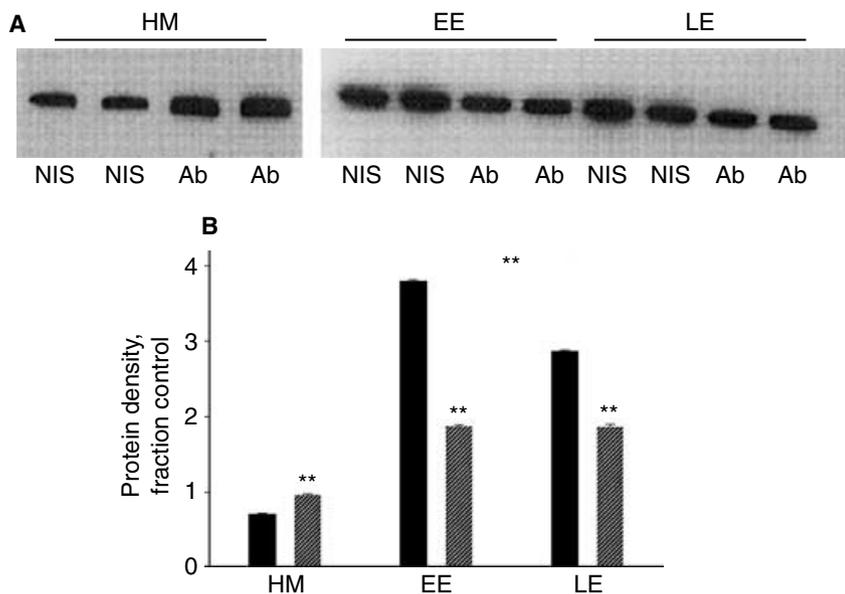
**Fig. 5. Dietary sodium intake and urinary sodium excretion rate (UNaV) in rats fed a high salt diet treated with either anti-MBG antibody or non-immune serum.** Data expressed as mean  $\pm$  SEM of 9 measurements in each group. Baseline data expressed as two days (baseline 1) and 1 day (baseline 2) prior to administration of antibody or nonimmune serum. Day 1 refers to the 24-hour period after the first dose of antibody or nonimmune serum, whereas day 2 refers to the 24-hour period following the second dose of antibody or nonimmune serum. \*\* $P < 0.01$  vs. baseline 1.



**Fig. 6. Effect of anti-MBG antibody or nonimmune serum administration on proximal tubular ouabain-sensitive  $^{86}\text{Rb}$  uptake (A) and Na/K-ATPase enzymatic activity (B) in rats fed a high salt diet.**  $^{86}\text{Rb}$  uptake data referenced to proximal tubules isolated from rats fed a normal salt diet (see Fig. 1A). Data expressed as mean  $\pm$  SEM of 5 determinations in each group. \*\* $P < 0.01$  vs. nonimmune serum.

of MBG in the Sprague-Dawley rat, an outbred strain known not to be salt sensitive in terms of blood pressure in contrast to the Dahl salt-sensitive strain, which is both inbred and well known to be quite salt sensitive with respect to blood pressure [9, 41]. We also observed that redistribution of the Na/K-ATPase appears to be taking place in response to the high salt diet in proximal tubule cells. Evidence to support this includes reduced  $^{86}\text{Rb}$  uptake and plasmalemmal Na/K-ATPase  $\alpha 1$  protein associated with a high salt diet along with substantial increases in both early and late endosomal Na/K-ATPase  $\alpha 1$  protein. We should also mention that while total prox-

imal tissue content of the Na/K-ATPase  $\alpha 1$  protein was unchanged by the high salt diet, enzymatic activity was decreased, suggesting that some modification of the protein, such as the binding of a cardiac steroid, occurred during this redistribution process. While we have demonstrated that endocytosis of the Na/K-ATPase stimulated by ouabain in LLC-PK1 cells appears to require clathrin-dependent pathways [23], our similar observations in the proximal tubules of salt loaded animals presented in this report, namely accumulation of the Na/K-ATPase in the endosomal fractions along with depletion from the plasmalemma, suggest that a similar process does occur in



**Fig. 7. Effect of anti-MBG antibody (Ab) or nonimmune serum (NIS) administration on heavy membrane [reflecting plasmalemma, early (EE) and late (LE) endosomal Na/K-ATPase  $\alpha 1$  content in rats fed a high salt diet].** (A) Shown are representative Western blots; the heavy membrane gels were loaded with 3  $\mu$ g, and the endosomal lanes were loaded with 25  $\mu$ g in each lane. Again, these amounts of proteins produced results within a linear range. (B) NIS shown as black bars, Ab shown as light gray bars. Data shown as fraction of value seen with control (0.4% NaCl) diet, and shown as mean  $\pm$  SEM. \*\* $P < 0.01$  vs. NIS. Heavy membrane early and late endosomal Na/K-ATPase  $\alpha 1$  content from proximal tubules of rats treated with non-immune serum did not differ significantly from that seen with high salt diet alone ( $N = 4$  comparisons in each group).

vivo. While endocytosis of the Na/K-ATPase occurs in response to other pharmacologic stimuli, such as dopamine [24, 27, 40], our study did not address how cardiotoxic steroid-stimulated endocytosis might interact with these other pathways.

We observed that administration of a polyclonal antibody to MBG, which has previously been demonstrated to bind circulating MBG and to markedly decrease urinary MBG excretion and free plasma MBG concentrations [9], blocked the proximal tubule Na/K-ATPase endocytosis stimulated by the high salt diet. Evidence for this assertion was the complete normalization of the proximal tubule  $^{86}\text{Rb}$  uptake, and substantial increase in Na/K-ATPase enzymatic activity coincident with a marked (60%) attenuation of natriuresis seen in rats fed a high salt diet. The discrepancy between increases in  $^{86}\text{Rb}$  uptake and Na/K-ATPase activity following antiserum may be due to simple addition of errors inherent in these measurements. However, it might also indicate that antibody binding to MBG (and/or other cardiotoxic steroids) occurs at a tissue level. We also observed that the administration of anti-MBG antibody (but not nonimmune serum) substantially reduced the early and late endosomal accumulation of the Na/K-ATPase seen with the high salt diet, and returned the plasmalemmal Na/K-ATPase density to that seen with the control (0.4% NaCl) diet. Although antibody-associated changes in blood pressure and/or glomerular filtration rate were not examined directly in our studies, we chose a non-salt-sensitive strain of rat, and used nonimmune serum to minimize and control for these potential effects. Further studies examining crosstalk between the dopaminergic and DLS stimulated systems will be necessary to determine the degree of overlap between these pathways. Moreover, we must point out that the implications of changes in Na/K-ATPase ex-

pression in proximal tubule cells on sodium handling are still unclear. Changes in the expression and function of the apical ion transporters may actually play a greater role in proximal tubular sodium transport than that of the basolateral Na/K-ATPase [43, 44]. Discussion of how cardiotoxic steroid-stimulated endocytosis of the Na/K-ATPase might affect apical ion transporter expression in the proximal tubule would be purely speculative at this point.

## CONCLUSION

Previous studies in a cell line model of proximal tubule cells suggested that cardiotoxic steroids could stimulate substantial redistribution of the plasmalemmal Na/K-ATPase. These studies demonstrate that such redistribution of the proximal tubule Na/K-ATPase also occurs in vivo, and that administration of antibodies to MBG, at least partially, reverses this process.

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