

## Contribution of the basolateral isoform of the Na-K-2Cl<sup>-</sup> cotransporter (NKCC1/BSC2) to renin secretion

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**Castrop, Hayo, John N. Lorenz, Pernille B. Hansen, Ulla Friis, Diane Mizel, Mona Oppermann, Boye L. Jensen, Josie Briggs, Ole Skøtt, and Jurgen Schnermann.** Contribution of the basolateral isoform of the Na-K-2Cl<sup>-</sup> cotransporter (NKCC1/BSC2) to renin secretion. *Am J Physiol Renal Physiol* 289: F1185–F1192, 2005. First published August 16, 2005; doi:10.1152/ajprenal.00455.2004.— Acute administration of loop diuretics like furosemide leads to a stimulation of renin secretion, an effect thought to result from inhibition of Na-K-2Cl cotransporter (NKCC2)-mediated salt transport at the luminal surface of the macula densa (MD). However, loop diuretics also inhibit NKCC1, the second isoform of the Na-K-2Cl cotransporter, with similar potency. In the present study, we examined the influence of furosemide on renin secretion in NKCC1-deficient mice to distinguish between effects of the loop diuretic involving NKCC2 and, by implication, the MD pathway, and effects that might occur via inhibition of NKCC1. Baseline plasma renin concentration (PRC) was  $1,212 \pm 211$  in NKCC1+/+ ( $n = 13$ ) and  $3,851 \pm 579$  ng ANG I·ml<sup>-1</sup>·h<sup>-1</sup> in NKCC1-/- mice ( $n = 14$ ;  $P = 0.00024$ ). Acute administration of furosemide (50 mg/kg i.p.) increased PRC significantly to  $9,324 \pm 1,018$  ng ANG I·ml<sup>-1</sup>·h<sup>-1</sup> in NKCC1+/+ ( $n = 13$ ;  $P < 0.0001$  compared with basal) and to  $14,188 \pm 2,274$  ng ANG I·ml<sup>-1</sup>·h<sup>-1</sup> in NKCC1-/- mice [ $n = 14$ ;  $P = 0.0002$  compared with basal;  $P = 0.034$  compared with wild-type (WT) plus furosemide]. Renin mRNA expression was about threefold higher in NKCC1-/- compared with WT mice. There was considerable recruitment of granular cells to upstream regions of afferent arterioles in NKCC1-/- mice. Patch-clamp studies in single juxtaglomerular granular (JG) cells from WT mice showed an ~10% increase in membrane capacitance during incubation with furosemide ( $10^{-4}$  M), indicating a direct effect of the loop diuretic on renin secretion. No effect of furosemide on membrane capacitance was observed in JG cells from NKCC1-deficient mice. Furosemide ( $10^{-3}$  M) significantly stimulated renin release from primary cultures of JG cells from WT mice, whereas no response was observed in NKCC1-/- mice. Our data suggest that a functional NKCC1 suppresses basal renin release, at least in part, through a direct effect on JG cells.

NKCC1 knockout mice; plasma renin; renin mRNA; patch clamp; juxtaglomerular granular cells

NaCl concentration in the tubular fluid in the tubulovascular contact region of the distal nephron has been shown to exert major regulatory effects on both preglomerular resistance and renin secretion (30). The interaction between tubules and vessels is thought to be initiated by the specialized epithelial cell plaque called the macula densa (MD), located at the distal end of the loop of Henle. This group of cells acts as a

chemosensor for luminal NaCl concentration: a low NaCl concentration in the tubular lumen generates a signal that leads to the release of renin from juxtaglomerular cells and to a dilatation of the smooth muscle cells of the afferent arteriole, whereas a high NaCl concentration has the opposite effects. Tubular NaCl concentration is believed to be detected as some direct function of the transport activity of the Na-K-2Cl cotransporter NKCC2 in the apical membrane of MD cells (21, 29). Much of the evidence supporting the role of NKCC2-mediated transport activity in MD signaling is derived from studies of the effects of inhibitors of NKCC2 such as furosemide or bumetanide. In fact, administration of loop diuretics has become a whole animal approach to determine the functional characteristics of MD control of both preglomerular resistance and renin secretion.

Nevertheless, the mechanisms responsible for the effects of systemic administration of loop diuretics are not entirely clear. These agents can inhibit both isoforms of the cotransporter, NKCC1 and NKCC2, with similar potency (13). The NKCC1 subtype of the Na-K-2Cl cotransporter is widely expressed in both epithelial and nonepithelial cells (11). In secretory epithelia, NKCC1 is typically found in a basolateral position. NKCC1 expression in the kidney is primarily localized in the inner medullary collecting duct but has also been found in cells of the afferent arteriole and extraglomerular mesangium (20). NKCC1 mRNA was detected by RT-PCR in various nephron segments, most abundantly in outer and inner medullary collecting ducts (16). Furthermore, in a recently developed cell line with MD characteristics, NKCC1 was found to be the predominant isoform of the cotransporter (14, 35). In contrast to the extended presence of NKCC1, expression of NKCC2 is restricted to the apical membrane of thick ascending limbs and MD cells (19, 34). Because of its conspicuous immunopositivity in the region of the juxtaglomerular apparatus, a role of NKCC1 in renin secretion seemed conceivable. Therefore, systemic administration of furosemide may exert effects that reflect inhibition of NKCC1 in addition, or as an alternative, to its action as inhibitor of NKCC2 activity in the loop of Henle and MD cells.

The present study was undertaken to explore the possibility that NKCC1 plays a role in basal renin release and in the stimulation of renin secretion known to be exerted by loop diuretics. Because of the absence of selective inhibitors of the NKCC isoforms, we utilized experimental models in which

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only one of the cotransporters is expressed so that the target of the loop diuretic is better defined. Our specific *in vivo* approach was to examine the renin-regulatory effect of furosemide in NKCC1 knockout mice in which the diuretic can only act through NKCC2 (5, 6). In addition, we used isolated juxtaglomerular granular cells to determine whether furosemide can affect renin secretion in the absence of the MD, an effect presumably mediated by inhibition of NKCC1.

Our studies in NKCC1<sup>-/-</sup> mice show that basal levels of renal renin mRNA were elevated compared with wild-type mice, and this was accompanied by an increase in renin expression that included recruitment of renin-forming cells outside the juxtaglomerular region. Furthermore, in isolated granular cells from mouse kidneys, furosemide caused an increase in membrane capacitance ( $C_m$ ), an index of renin exocytosis, in cells from wild-type, but not NKCC1<sup>-/-</sup>, mice. These results suggest that renin synthesis and secretion are tonically suppressed at the level of the renin-generating cells by NKCC1-mediated NaCl flux.

## METHODS

**Animals.** NKCC1-deficient mice (NKCC1<sup>-/-</sup>) and their wild-type controls were from a colony originally generated by Flagella et al. (6) and maintained at the University of Cincinnati. Animal care and experimentation were approved and carried out in accordance with institutional guidelines and National Institutes of Health principles in their *Guide for the Care and Use of Laboratory Animals*. An additional group of 10 NKCC1<sup>-/-</sup> and 10 wild-type mice from a subcolony of the original knockout strain maintained at Emory University was used for the blood pressure and *in vitro* studies.

**Blood collection and renin determination.** Tail blood was taken from conscious mice by nicking the tail with a razor blade and collecting the emerging blood into a 75- $\mu$ l hematocrit tube that contained 1  $\mu$ l 125 mM EDTA in its tip. Red cells and plasma were separated by centrifugation; the plasma was ejected into an Eppendorf tube and frozen until used for renin determinations. Plasma renin concentration (PRC) was measured with a commercial radioimmunoassay kit (Gammacoat, DiaSorin, Stillwater MN). In our approach, 1  $\mu$ l of a 1:5 plasma dilution (equivalent to 0.2  $\mu$ l of undiluted plasma) was mixed on ice with 14  $\mu$ l of dialyzed rat substrate and 10  $\mu$ l of maleate buffer containing EDTA, neomycin sulfate, and PMSF provided by the manufacturer. For determination of background angiotensin I levels, 10  $\mu$ l of this mix were removed and kept frozen until assaying. The remaining aliquot was incubated for 1 h at 37°C. Generated angiotensin I was measured by radioimmunoassay using standards and reagents provided by the manufacturer. In each assay, substrate without plasma was incubated for the same time, and any background angiotensin I formation was subtracted from the plasma-containing samples. Angiotensin I levels determined in the nonincubated plasma aliquot were also subtracted. The high renin levels in the mouse especially after furosemide treatment pose a problem for measurements of PRC because saturating amounts of substrate are difficult to maintain for the entire incubation period. The amount of substrate used in these studies is based on pilot experiments aimed at establishing saturation conditions for plasma samples with very high renin levels.

For basal renin determinations, all animals were kept in individual cages at least 3 days before blood collections. Separation of mice, especially of male animals, was found to reduce data variation and to minimize baseline renin values (2). Following baseline blood collections, animals were allowed to recover for 3 days before they received a single injection of furosemide (50 mg/kg ip; Lasix, Hoechst). Blood was collected 45 min later, whereas urine was collected over this period in metabolic cages to verify the efficacy of the injection.

**Visualization of JG cell granules.** Kidneys from NKCC1<sup>-/-</sup> and <sup>+/+</sup> mice were treated with 5 M HCl for 1 h at 37°C. After acid removal, kidneys were kept in distilled water for 48 h at 4°C. Vascular trees were microdissected under a stereomicroscope, and the JG cells were directly visualized as described by Casellas et al. (1). In four vessel trees from each genotype, we counted the number of afferent arterioles that showed typical juxtaglomerular granularity, additional upstream recruitment, or no granularity.

**Real-time PCR.** Total RNA was extracted from whole kidney using TRIzol reagent and denatured at 70°C for 5 min. cDNA synthesis was done at 42°C for 45 min using superscript RT. For real-time PCR, renin primers were chosen by using PRIMER EXPRESS 2.0 (PE Applied Biosystems) with an MGB probe being positioned at an exon-intron boundary. The primer/MGB probe mixture for 18S rRNA was purchased from Applied Biosystems as was the  $\beta$ -actin primer/probe set. Real-time PCR amplification was performed in 50 ng cDNA using TaqMan Universal PCR Master Mix. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 repeats at 95°C for 0.15 min and 60°C for 1 min. The relative amount of renin mRNA, normalized by 18S rRNA or  $\beta$ -actin, was expressed as  $2^{-\Delta\Delta CT}$  calculated from threshold cycle numbers (CT) according to the manufacturer's suggestion.

**Isolation of JG cells and renin secretion studies.** Kidneys from C57BL/6 mice (wild-type) and NKCC1<sup>-/-</sup> mice were removed, decapsulated, minced, and transferred to 30 ml of isolation buffer supplemented with 0.1% (wt/vol) of collagenase A (0.57 U/mg; Roche Diagnostics) and 0.25% (wt/vol) of trypsin [1,300 U benzyl-L-arginine ethyl ester (BAEE)/mg; Sigma]. The tissue was incubated and stirred gently for 70 min at 37°C, then filtered through 22- $\mu$ m nylon mesh. The filtrate was washed, centrifuged, and resuspended in 4 ml of isolation buffer. For patch-clamp studies, JG cells were identified by their appearance and transferred individually to coverslips. For renin secretion studies, cells were further separated on a Percoll density gradient (25% Percoll) by centrifugation for 30 min at 27,000 g (4°C). Four cell layers with different specific renin activities were obtained. The cellular layer (equivalent to a density of 1.049 g/ml) with the highest renin concentration (100-fold increase in specific renin activity) was used for the experiments. These cells were washed twice and resuspended in RPMI-1640 medium to a concentration of 150,000 cells/ml. Media contained FCS (2%), insulin, penicillin, and streptomycin. Aliquots (100  $\mu$ l) of this suspension were seeded in 96-multiwell plates. After the cells were incubated for 20 h, they were washed and experimental agents [furosemide ( $10^{-5}$  to  $10^{-3}$  M), forskolin ( $10^{-5}$  M)] were added. Cell-conditioned medium was removed after another 20 h, centrifuged at 10,000 g at room temperature to remove cellular debris, and renin concentration was determined by radioimmunoassay of angiotensin I (18). The cells were lysed by the addition of 100  $\mu$ l of PBS with 0.1% of Triton X-100 and 0.1% human serum albumin to each well as described by Friis et al. (7). The plates were shaken for 45 min at room temperature, and lysates were centrifuged at 10,000 g for 10 min. The supernatants were stored at -20°C until further processing. The renin concentration in the supernatants was measured after incubation for 3 h with excess rat renin substrate followed by RIA against angiotensin I. Renin secretion rates were calculated as fractional release of total renin content [i.e., renin released/(renin released + renin remaining in the cells)].

**Patch-clamp experiments.** A glass coverslip with individual JG cells was superfused with the control external solution, then transferred to the recording chamber and supplemented with buffer to a volume of ~400  $\mu$ l. Experiments were performed at room temperature in the tight-seal, whole-cell configuration of the patch-clamp technique using heat-polished, Sylgard-coated patch pipettes with resistances of 3–7 M $\Omega$ . Series resistances were in the range of 6–25 M $\Omega$ , and seal resistances were in the range of 1–15 G $\Omega$ . High-resolution membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled

by PULSE v8.11 software on a Power Macintosh G3 computer. High-resolution currents were low-pass filtered at 2.9 kHz and acquired at a sampling rate of 20 kHz. The current-voltage relationship (*I-V*) was monitored by the response to nine voltage steps of 30 mV (range  $-150$  to  $+90$  mV) for 200 ms from a holding potential of  $-30$  mV. Low-time-resolution acquisition of  $C_m$  was performed with the "sine+dc" method using the LockIn extension of the PULSE v8.11 software. The  $C_m$  measurements were started no later than 30 s after the *I-V* recording. Data from an entire sweep were averaged to result in 1  $C_m$  point/sweep, resulting in an acquisition rate of  $\sim 5$  Hz using the Xchart extension of the PULSE software. The reference electrode was an Ag/AgCl pellet connected to the bath solution through a 150 mmol/l NaCl-agar bridge. All potentials were corrected for the liquid junction potential that develops at the tip of the pipette when it is immersed in the bath solution. The following solutions were used: 1) an "internal" control solution for patch clamp (in mmol/l): 135 K-glutamate, 10 NaCl, 10 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 0.5 Mg-ATP, and 0.3 Na<sub>2</sub>GTP, with osmolality of 306 mosmol/kgH<sub>2</sub>O and pH 7.05 (22°C); and 2) an "external" (bath) solution for patch clamp (in mmol/l): 10 HEPES, 140 NaCl, 2.8 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 11 glucose, and 10 sucrose, with osmolality of 304 mosmol/kgH<sub>2</sub>O and pH 7.21 (25°C).

**RT-PCR in single JG cells.** Single mouse JG cells were used to determine the presence of NKCC1 mRNA by RT-PCR. Twenty cells were selected one by one using a patch pipette, and RNA was isolated according to a microadaptation of the protocol of Chomczynski and Sacchi (3). PCR amplification was performed for 36 cycles using 3.3  $\mu$ l of cDNA equivalent to 2.5 JG cells. Negative controls included water instead of cDNA in the PCR reaction and  $-RT$ . As a positive control, the kidney medulla was used as a template. The published sequence for mouse NKCC1 (NM\_009194) was used to design primers for PCR amplification, and the primer sequence was as follows: NKCC1: sense 5'-GAA CCT TTT GAG GAT GGC-3', antisense 5'-CAC GAT CCA TGA CAA TCT-3' covering base pair numbers 865-1044 and resulting in a product length of 179 +15 (restriction site), i.e., 194 bp.

**Blood pressure and heart rate.** Systolic blood pressures and heart rates of six wild-type and five NKCC1-deficient mice were determined by tail-cuff manometry (Visitech Systems). Animals were conditioned by placing them into the holding device on 3 consecutive days before the first measurement. Blood pressure was determined for 3 days in a row, and values were calculated as averages of these three measurements for each individual mouse.

**Statistics.** All values are means  $\pm$  SE. ANOVA was performed to test for statistical significance of differences among the values observed in each treatment group followed by a Bonferroni posttest. A *P* value  $<0.05$  was considered significant.

## RESULTS

**PRC in NKCC1<sup>+/+</sup> and NKCC1<sup>-/-</sup> mice.** Baseline PRC averaged  $1,212 \pm 211$  in NKCC1<sup>+/+</sup> ( $n = 13$ ) and  $3,851 \pm 579$  ng ANG I $\cdot$ ml<sup>-1</sup> $\cdot$ h<sup>-1</sup> in NKCC1<sup>-/-</sup> mice ( $n = 14$ ;  $P = 0.00024$  compared with wild-type). Measurements in individual mice are shown in Fig. 1. After a single intraperitoneal injection of furosemide (50 mg/kg), PRC increased to  $9,324 \pm 1,018$  ng ANG I $\cdot$ ml<sup>-1</sup> $\cdot$ h<sup>-1</sup> in wild-type animals ( $n = 13$ ;  $P < 0.0001$ ) and to  $14,188 \pm 2,274$  ng ANG I $\cdot$ ml<sup>-1</sup> $\cdot$ h<sup>-1</sup> in NKCC1<sup>-/-</sup> mice ( $n = 14$ ;  $P = 0.0002$  compared with basal and  $P = 0.034$  vs. wild-type). Thus the furosemide effect consisted of a PRC increase of  $8,112 \pm 930$  ng ANG I $\cdot$ ml<sup>-1</sup> $\cdot$ h<sup>-1</sup> in wild-type and  $11,118 \pm 2,208$  ng ANG I $\cdot$ ml<sup>-1</sup> $\cdot$ h<sup>-1</sup> in NKCC1<sup>-/-</sup> mice ( $P = 0.23$ ), representing a  $9.9 \pm 1.5$ -fold rise in wild-type and a  $4.4 \pm 0.7$ -fold rise in NKCC1<sup>-/-</sup> mice ( $P = 0.003$ ).

**Visualization of granular cells.** The method of Casellas et al. (1) was used to visualize and quantify the extent of granulation in afferent arterioles. As has been documented previously, the high-contrast regions usually seen at the end of afferent arterioles represent clusters of renin-positive cells (1). It is evident from the examples shown in Fig. 2 that the extent of granulation is clearly augmented in the vascular trees from NKCC1<sup>-/-</sup> kidneys. Furthermore, vessels from NKCC1-deficient mice show high-contrast areas in upstream regions of the afferent arterioles representing recruitment of renin-producing cells outside the juxtaglomerular location. In vessel trees from wild-type mice, quantification in 4 vessel trees with a total of 110 arterioles showed that  $52.9 \pm 2\%$  of all vessels had renin positivity at the terminal ends of the arterioles whereas  $47.1 \pm 2\%$  were negative. In contrast, analysis of 4 vessel trees from NKCC1<sup>-/-</sup> kidneys with 163 identifiable arterioles showed recruitment to upstream regions in  $73.1 \pm 3.9\%$  of the vessels whereas exclusive "classic" JG positivity was seen in  $22.4 \pm 5\%$ , with only  $4.5 \pm 1.5\%$  of arterioles being negative.

**Renin mRNA.** Renin mRNA was determined by real-time PCR in five kidneys from wild-type mice and five kidneys from NKCC1<sup>-/-</sup> mice. Relative amounts of renin mRNA in wild-type mice, expressed as  $2^{-\Delta\Delta CT}$  values calculated from CT numbers, averaged  $1.51 \pm 0.6$  when corrected for 18S rRNA and  $1.42 \pm 0.4$  when corrected for  $\beta$ -actin. As shown in Fig.

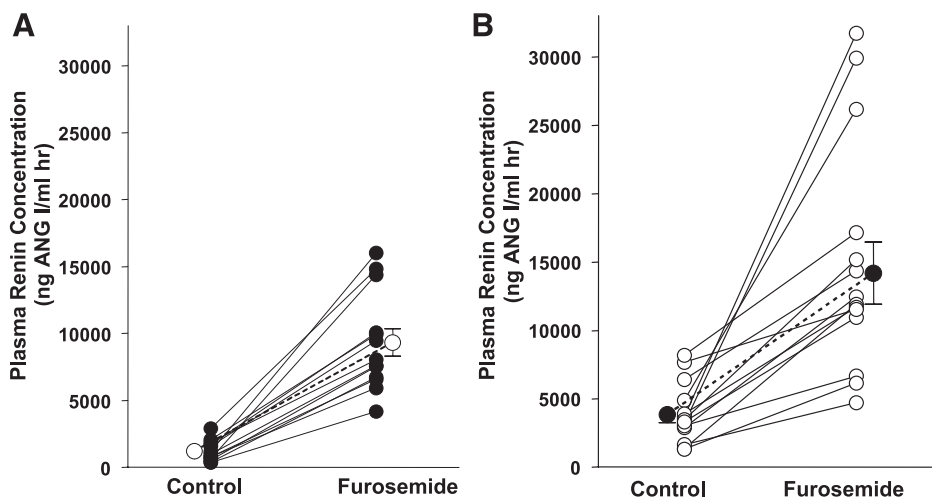


Fig. 1. Plasma renin concentration (PRC) in NKCC1<sup>+/+</sup> (A) and NKCC1<sup>-/-</sup> mice (B) under basal conditions and after acute administration of furosemide (50 mg/kg). Lines connect data from individual animals. Bold symbols and connecting lines represent means.



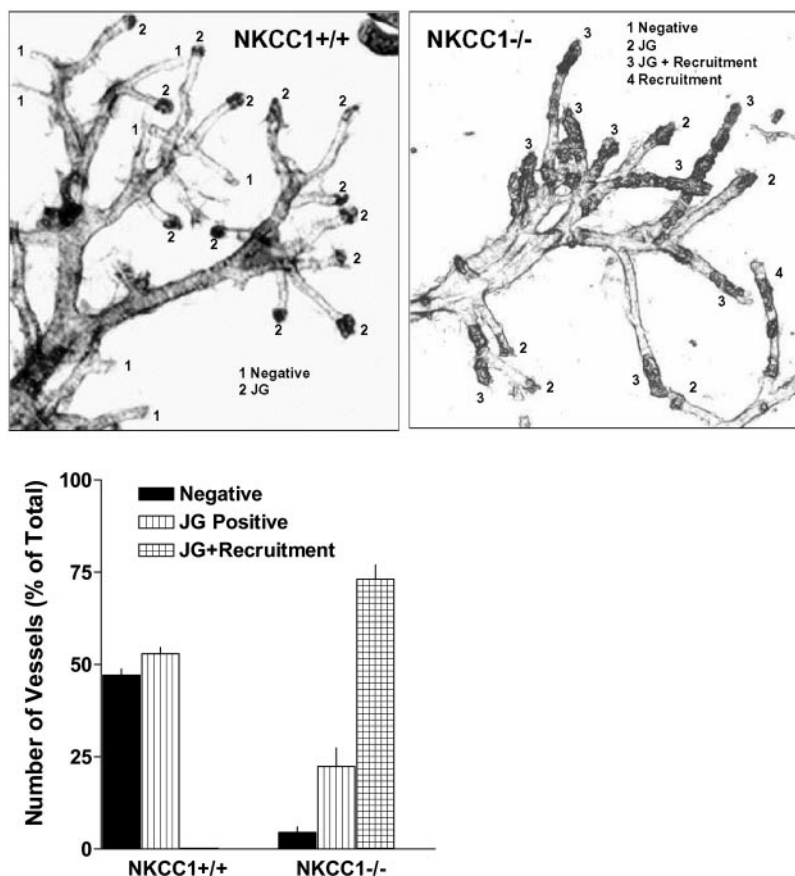


Fig. 2. *Top*: visualization of renin granules after acid maceration of microdissected renal arterial vessel trees from a NKCC1<sup>+/+</sup> (left) and a NKCC1<sup>-/-</sup> mouse (right). High-contrast regions at the end of the arterioles represent clusters of renin granules (glomeruli are sheared off in the microdissection process). *Bottom*: count of negative, juxtaglomerular granular (JG)-positive, and JG-positive afferent arterioles with upstream recruitment.

3, renin mRNA levels in kidneys of NKCC1<sup>-/-</sup> mice were three times higher than in control when corrected for 18S rRNA ( $4.57 \pm 0.57$ ;  $P = 0.0006$  compared with wild-type), and 2.2 times higher than in control when corrected for  $\beta$ -actin ( $3.2 \pm 0.3$ ;  $P = 0.004$  compared with wild-type).

**Effect of furosemide on renin secretion from isolated JG cells in primary culture.** Experiments were performed in isolated JG cells to investigate a possible direct effect of furosemide on renin secretion. In four independent batches of JG cells harvested from wild-type and NKCC1<sup>-/-</sup> mice, fractional renin release under baseline conditions averaged  $20.6 \pm$

$1.6\%$  ( $n = 5$ ) in wild-type compared with  $36.9 \pm 2.2\%$  ( $n = 4$ ) in NKCC1<sup>-/-</sup> mice. After incubation with furosemide, mean fractional renin release was  $22.6 \pm 2.1$ ,  $27.7 \pm 3.0$ , and  $31.7 \pm 3.4\%$  for  $10^{-5}$ ,  $10^{-4}$  and  $10^{-3}$  M furosemide, respectively, with differences reaching the 5% level of significance at a furosemide concentration of  $10^{-3}$  M in wild-type mice (Fig. 4). In this preparation, the well-known stimulator of renin release, forskolin, at a concentration of  $10^{-5}$  M increased fractional renin release to  $35.9 \pm 2.4\%$ . As can also be seen in Fig. 4, furosemide had no effect on mean fractional renin release in cells from NKCC1<sup>-/-</sup> mice, averaging  $39.3 \pm 1.6$ ,  $40.4 \pm 1.6$ , and  $37.7 \pm 2.0\%$  for  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M furosemide, respectively. Forskolin increased renin release to  $52.2 \pm 1.4\%$  in NKCC1<sup>-/-</sup> mice.

**Patch-clamp experiments.** Because primary cultures of JG cells isolated by gradient centrifugation are to some extent contaminated by other cell types, we measured the change in  $C_m$  of single JG cells from wild-type and NKCC1-deficient mice in response to furosemide by patch-clamp in the whole cell configuration as a measure for secretion. As shown in Fig. 5A for a single trace, in JG cells from wild-type mice furosemide ( $10^{-4}$  M) led to a gradual increase in  $C_m$  over a period of 1,200 s, corresponding to secretory activity. As can be seen in Fig. 5B, the average change in  $C_m$  after 1,200 s equaled  $+8.1 \pm 0.9\%$  ( $P < 0.05$ ,  $n = 5$ ) in JG cells from wild-type and  $-0.53 \pm 3.3\%$  in JG cells from NKCC1<sup>-/-</sup> mice ( $P = 0.28$ ,  $n = 4$ ). Figure 5C shows the  $I-V$  curves for JG cells from wild-type and NKCC1<sup>-/-</sup> mice recorded before and after superfusion with furosemide. Nine pulses were applied from

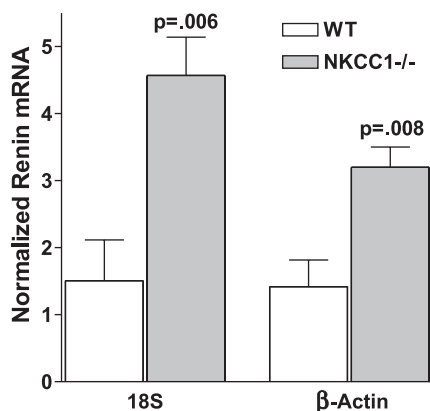


Fig. 3. Basal renin mRNA from kidneys of NKCC1<sup>+/+</sup> and NKCC1<sup>-/-</sup> mice ( $n = 5$  each) determined by real-time RT-PCR. Values are given after normalization with 18S rRNA or  $\beta$ -actin, respectively. WT, wild-type.

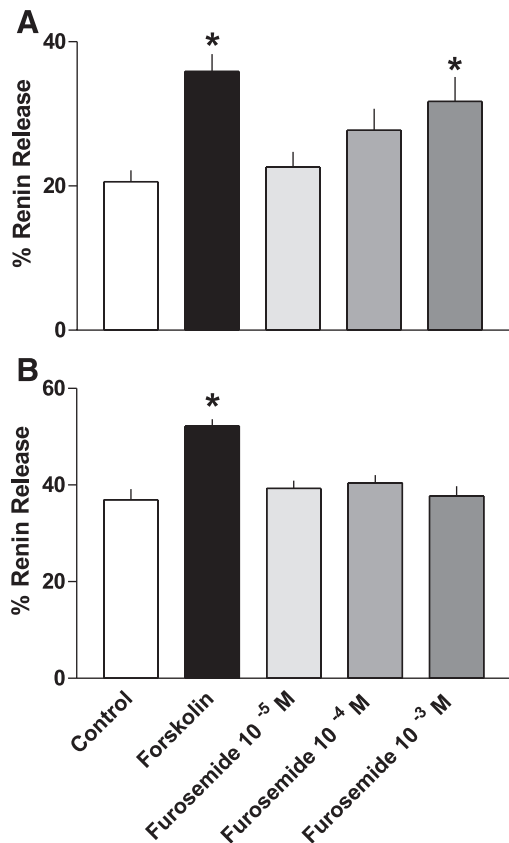


Fig. 4. Fractional renin release in preparations of isolated JG cells in primary culture. Basal renin release is compared with the effect of forskolin ( $10^{-5}$  M) and to that of increasing concentrations of furosemide for JG cells from wild-type (A) and NKCC1-deficient mice (B). \* $P < 0.05$  vs. control.

–150 to +90 mV in 30-mV steps for 200 ms from a holding potential of –30 mV. *I-V* was measured immediately after the whole cell configuration was obtained and after 20 min of capacitance recording in the presence of furosemide ( $10^{-4}$  M) during the last 14 min. A large increase in the outward current during superfusion with furosemide in JG cells from wild-type mice can be noted. The maximal outward current at +90 mV amounted to  $469 \pm 139$  pA at the beginning of the experiment without furosemide and  $1,118 \pm 329$  pA after 14 min of superfusion with furosemide ( $10^{-4}$  M) for JG cells from NKCC1+/+ ( $P < 0.05$ ;  $n = 5$ ), and  $466 \pm 65$  and  $512 \pm 82$  pA, respectively, for JG cells from NKCC1–/– mice ( $P = 0.09$ ;  $n = 4$ ). The increase in outward current in wild-type JG cells is also reflected as a hyperpolarization of the membrane potential amounting to  $-14.2 \pm 6.6$  mV ( $P < 0.05$ ,  $n = 5$ ). In a control experiment, intracellular application of cAMP increased both  $C_m$  and outward current in JG cells from both NKCC1+/+ and NKCC1–/– mice. The outward current was probably carried by  $K^+$  through  $BK_{Ca}$  channels of the ZERO-splice variant (10).

**NKCC1 expression in single JG cells.** In cDNA prepared from single isolated JG cells, PCR amplification with primers specific for NKCC1 consistently yielded a PCR product of the expected size (194 bp). An example is shown in Fig. 6. A band of this size was also detected in cDNA from kidney medulla used as a positive control, whereas no band was obtained in the –RT and water controls.

**Blood pressure and heart rate in NKCC1+/+ and NKCC1–/– mice.** In view of the substantial basal activation of the renin system in NKCC1-deficient mice, blood pressure was determined in NKCC1+/+ and –/– mice by tail-cuff manometry. Systolic blood pressure averaged  $122 \pm 3.4$  in wild-type ( $n = 6$ ) and  $114 \pm 3.3$  mmHg in NKCC1–/– mice ( $n = 5$ ;  $P = 0.13$ ). Heart rate was  $588 \pm 17$  in wild-type and  $537 \pm 29$  beats/min in knockout mice ( $P = 0.17$ ).

## DISCUSSION

Furosemide-stimulated renin secretion is widely thought to affect renin-secreting granular cells indirectly through modulation of NKCC2-dependent NaCl transport in MD cells (29, 31). The present studies were performed with the specific aim to examine whether the stimulatory effect of loop diuretics on renin release includes a component that may be mediated by a direct interaction with JG cells and may therefore be due to inhibition of NKCC1. Because specific inhibitors of the two NKCC isoforms are not available, we made an attempt to examine the effect of loop diuretics in experimental models in which only one of the two isoforms was present.

The first goal of these studies was to compare the effect of furosemide on plasma renin concentration in wild-type and NKCC1-deficient mice (6). Our data show that basal PRC of NKCC1–/– mice is significantly elevated, by a factor of ~3.2, compared with wild-type controls and that this increase in PRC is paralleled by a marked elevation of renal renin mRNA and protein expression. These observations suggest that the net effect of a functional NKCC1 is to tonically suppress renin synthesis and renin release. It is appreciated that these *in vivo* experiments do not permit a clear distinction between direct and indirect consequences of the NKCC1 null mutation on the renin-angiotensin system. In particular, the possibility needs to be considered that the stimulation of renin is the result of the reduced arterial blood pressure that has been observed in NKCC1–/– mice (6, 23). However, in another strain of NKCC1 null mutant mice blood pressure tended to be lower in the null animals, but this change did not reach statistical significance (28). Measurements of blood pressure with the tail-cuff method in our laboratory also showed a tendency toward lower systolic blood pressures in NKCC1-deficient mice, but again this difference was not significant statistically. In view of these variable findings even in the same strain of NKCC1 mice, we assume that differences in blood pressure observed by different groups might not reflect actual differences in baseline blood pressure, but rather may be related to differential blood pressure responses to stressful interventions in these animals. The 3.2-fold difference in basal PRC between wild-type and NKCC1–/– mice observed in our study seems somewhat disproportionate to a systolic blood pressure reduction that appears to be only marginally significant. Thus while a reduction in blood pressure may be a contributory factor to the elevated PRC in NKCC1–/– mice, we consider it unlikely that it is the main reason. In addition to the absence of a significant change in blood pressure in these studies, the *in vitro* evidence discussed below clearly supports the likelihood of a direct, pressure-independent effect of NKCC1.

There is little reason to assume that the increase in PRC in the NKCC1–/– mice could be the result of an activation of the MD pathway. NKCC1–/– mice do not appear to be volume

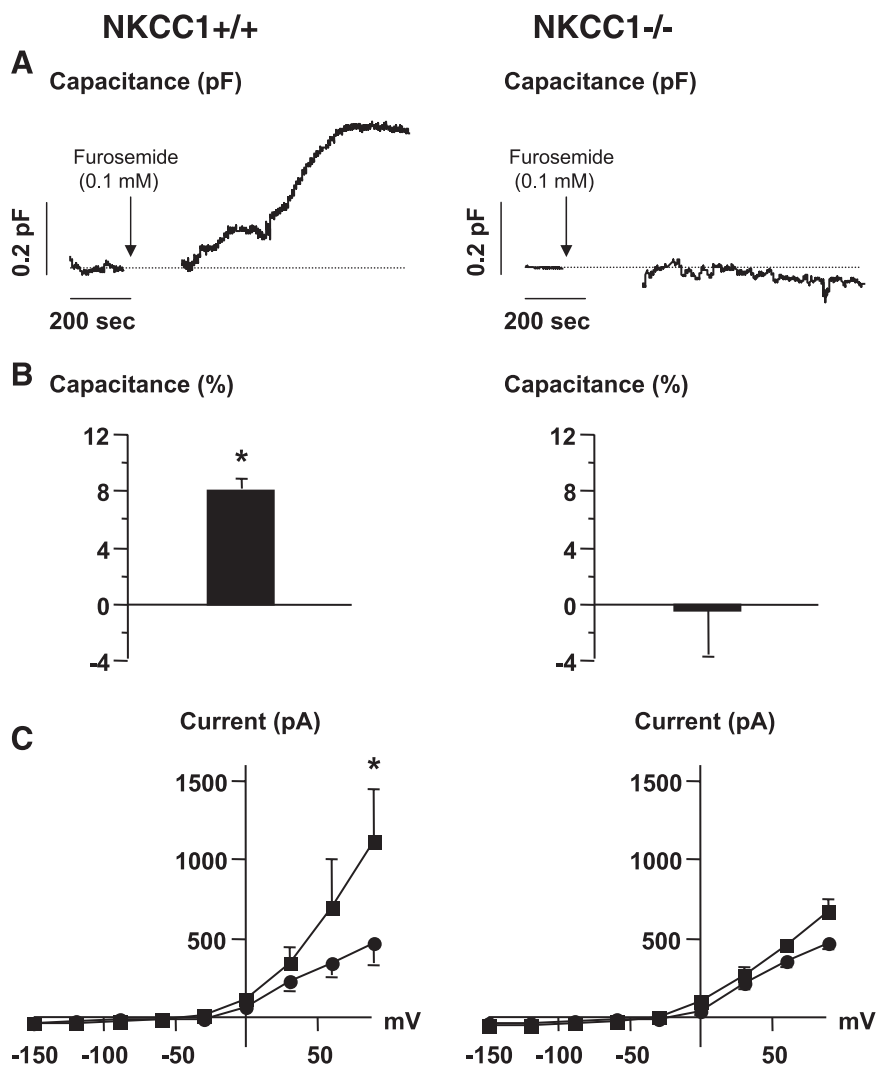


Fig. 5. A: original recording of membrane capacitance in a single mouse juxtaglomerular cell from NKCC1+/+ (left) and NKCC1-/- mice (right). The pipette contained control internal solution, and the cell was bathed in control external solution supplemented with furosemide (10<sup>-4</sup> M) at the time indicated by the arrow (see METHODS). B: mean relative change of membrane capacitance for JG cells from NKCC1+/+ (left) and NKCC1-/- mice (right). C: whole cell currents were measured in response to 9 pulses from -150 to +90 mV in 30-mV steps for 200 ms from a holding potential of -30 mV. These pulses were applied before and after superfusion with furosemide (10<sup>-4</sup> M). Mean steady-state current-voltage curves from 5 independent experiments before (●) and after (■) 20 min of capacitance measurements are given. \*P < 0.05 vs. control.

depleted, as judged from normal plasma aldosterone levels and the lack of effect of a low-Na diet on arterial blood pressure (23, 28). It is therefore unlikely that NaCl concentration in the MD region is markedly reduced. An absence of volume depletion also suggests that sympathetic input to the kidney is probably unaltered in NKCC1-/- mice. Finally, while NKCC1 has been found to be expressed in a cell line with MD

characteristics, there is little evidence that native MD cells express NKCC1 at detectable levels (14). Thus the stimulation of the renin system in NKCC1-/- mice probably does not result from a specific deficiency in the transporter in MD cells. In view of our observations in isolated JG cells, we believe that the increase in PRC in NKCC1-/- mice reflects disinhibition of renin release from tonic suppression exerted by NKCC1 at the level of the JG cells. It is of note that chronic NKCC1-/- deficiency is associated with a change in the expression pattern of renin that is characterized by a remarkable recruitment of renin-expressing cells outside the juxtaglomerular region. It is possible therefore that NKCC1 transport activity is related to suppression of renin expression in more proximal vascular cells and to the restriction of renin expression to the JG region. The release of renin by recruited cells may contribute to the higher levels of basal plasma renin in NKCC1-/- mice.

Direct evidence for an MD-independent renin-stimulatory effect of NKCC1 inhibition was derived from the effect of loop diuretics in primary cultures of JG cells isolated by gradient centrifugation methods. Basal renin release was higher in cells from NKCC1-/- than in those from wild-type mice. In addition, furosemide caused a significant increase in renin release in wild-type mice whereas it had no effect in

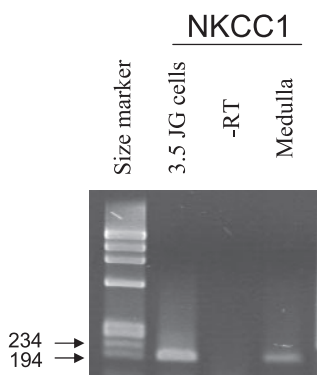


Fig. 6. RT-PCR on RNA prepared from single, isolated JG cells. cDNA from the renal medulla was used as a positive control.

NKCC1<sup>-/-</sup> mice. Compared with the reported IC<sub>50</sub> values of furosemide inhibition of NKCC1 or NKCC2, the concentrations of furosemide required to cause significant stimulation of renin release in the isolated JG cell preparation were relatively high. While this finding may suggest a nonspecific action of the diuretic, it is also possible that the inhibitory constant of furosemide is to some extent cell specific (13). Furthermore, the affinity of the diuretic to inhibit NKCC1 appears to depend on the activation state of the transporter. For example, an ~100-fold increase in the IC<sub>50</sub> concentration for furosemide has been observed in rat thymocytes under nonhypertonic conditions where the transporter activity is markedly reduced (13). Direct evidence for a specific effect of furosemide on NKCC1-mediated transport in our preparations is furnished by the finding that the diuretic did not alter renin secretion in JG cells from NKCC1-deficient mice.

Because JG cell preparations are usually somewhat contaminated with other cell types, we also assessed the effect of furosemide on renin secretion in single JG cells. Increases in  $C_m$  of single JG cells have previously been shown to occur with the administration of intracellular cAMP and extracellular isoproterenol as well as with cell swelling (7, 8). As previously demonstrated, cell  $C_m$  measured by patch-clamp techniques is a reflection of the secretory activity of a cell because exocytosis requires fusion events that increase membrane area and capacitance (24). Thus  $C_m$  changes in granular cells are very likely an expression of exocytosis of renin granules (8). Our data permit the conclusion that furosemide causes a significant increase in renin secretion in JG cells from wild-type mice that is comparable in magnitude to that produced by forskolin (7). In contrast to its effect in JG cells from wild-type mice, furosemide did not affect the capacitance of JG cells isolated from NKCC1-deficient mice. These observations provide clear evidence for the notion that furosemide stimulates the renin system by interacting with NKCC1 expressed in the membrane of JG cells, thereby eliminating a renin-suppressing effect of the NaCl transporter. Previous transporter localization studies have consistently failed to show NKCC2 or its mRNA outside the epithelium of the thick ascending limb (25, 26). On the other hand, the presence of NKCC1 in renin-positive cells of afferent arterioles has previously been suggested by immunohistochemistry (20). In addition, we could detect NKCC1 mRNA by RT-PCR in RNA isolated from single granular cells. On the basis of these observations, we believe that the effect of furosemide *in vivo* is to some extent the result of a direct interaction of the loop diuretic with NKCC1 in granular cells.

The patch-clamp studies also provide some insights into the mechanisms underlying the increase in renin secretion during blockade of NKCC1 activity. These experiments have shown that furosemide induced an increase in an outward current that was carried by potassium and caused marked cell hyperpolarization. Previous studies in isolated JG cells have shown that activation of the cAMP-sensitive ZERO splice variant of BK<sub>Ca</sub> is the likely cause for the hyperpolarization (10). However, it is unclear how furosemide causes an increase in cAMP that could explain its effect on membrane potential and renin release. Alternatively, a decreased intracellular Cl<sup>-</sup> concentration caused by inhibition of NKCC1-dependent chloride transport might either inhibit voltage-dependent calcium channels (27) directly or indirectly by driving HCO<sub>3</sub><sup>-</sup> out of the cell via the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger (33). Thus a resulting reduction in

cytosolic Ca in granular cells may be another pathway through which loop diuretics stimulate renin release (12). However, inactivation of L-type Ca channels may be an unlikely mechanism of action in view of the recent finding that these channels are essentially inactive at the membrane voltages observed in JG cells (9). Regardless of the exact mechanism, our studies in isolated JG cells indicate that NKCC1 transport activity suppresses basal renin synthesis and secretion and that this transport activity resides in the JG cells themselves.

A second goal of this study was to assess the possibility that the acute renin-stimulatory response to furosemide *in vivo* may be mediated by inhibition of both NKCC isoforms. Such dual actions of loop diuretics on renin secretion are suggested by earlier evidence. For example, in the isolated, perfused juxtaglomerular apparatus of the rabbit, stimulation of renin secretion has been observed when loop diuretics were administered into the tubular lumen, a situation where inhibition of extratubular NKCC1 may be unlikely (15, 17, 22). However, this increase in renin release amounted to only about threefold (22), much less than the about six- to eightfold increase caused by loop diuretics in this and several other *in vivo* studies. Furthermore, an action of furosemide on renin secretion through a MD- and therefore, presumably, NKCC2-independent, pathway has been suggested in nonfiltering kidneys (4, 32). Our results show that acute *in vivo* administration of furosemide increased PRC levels in wild-type mice by a factor of 9.9, whereas the relative increase over control was only 4.4-fold in NKCC1<sup>-/-</sup> mice. However, this reduction in the relative increase of PRC in NKCC1<sup>-/-</sup> mice appears to be due to the elevated baseline levels because the absolute increase in PRC caused by furosemide was not significantly different between wild-type and NKCC1<sup>-/-</sup> mice (8,112 vs. 11,118 ng ANG I·ml<sup>-1</sup>·h<sup>-1</sup>;  $P = 0.23$ ). Thus it appears that furosemide stimulates renin release to a comparable extent with or without NKCC1, suggesting that the acute effect of furosemide is largely the result of NKCC2 inhibition. It is conceivable that the absence of a demonstrable effect of furosemide on NKCC1 results from the fact that, because of tubular secretion, furosemide concentrations at any given plasma level will be higher at the MD so that NKCC2 is exposed to higher furosemide concentrations than NKCC1. However, at the dose of 50 mg/kg used in these studies one would expect all NKCC isoforms to be at least transiently inhibited although it is not certain that inhibition was sustained for the entire 45-min period. Nevertheless, because the effect of furosemide on PRC was comparable between wild-type and NKCC1-deficient mice, inhibition of NKCC1 does not appear to significantly contribute to the acute renin-stimulatory effect of furosemide. The newly recruited renin-producing cells that may be the main cause for the elevated basal renin levels of NKCC1<sup>-/-</sup> mice are not expected to respond to furosemide because they are presumably not under MD control and therefore not affected by NKCC2 inhibition.

In conclusion, our results show that basal plasma renin concentration is markedly elevated in NKCC1<sup>-/-</sup> compared with wild-type mice, suggesting tonic suppression of renin synthesis and secretion by NKCC1 transport activity. Patch-clamp studies in isolated granular cells have demonstrated the capacity of furosemide to stimulate renin exocytosis. Thus NKCC1 exerts its renin-inhibiting actions, at least in part, through a direct effect at the level of the JG cells.



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