Renal Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter activity and vasopressin-induced trafficking are lipid raft-dependent

Pia Welker,\textsuperscript{1*} Alexandra Böhlick,\textsuperscript{1*} Kerim Mutig,\textsuperscript{1} Michele Salanova,\textsuperscript{1} Thomas Kahl,\textsuperscript{1} Hartmut Schlüter,\textsuperscript{2} Dieter Blottner,\textsuperscript{1} Jose Ponce-Coria,\textsuperscript{3} Gerardo Gamba,\textsuperscript{3} and Sebastian Bachmann\textsuperscript{1}

1Department of Anatomy, Charité-Universitätsmedizin Berlin, Berlin; 2Department of Nephrology, Charité-Universitätsmedizin Berlin, Berlin, Germany; and 3Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico

Submitted 1 April 2008; accepted in final form 17 June 2008

The kidney-specific, apical Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter (NKCC2 or BSC1; SLC12A1) is strongly expressed on the luminal membrane of renal tubular cells of the thick ascending limb of Henle’s loop (TAL) (14, 32), where it is crucial for normal Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{−}, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+} reabsorption (16). It also serves to maintain the corticomedullary osmotic gradient that drives urinary concentration in response to the antidiuretic hormone vasopressin (AVP) (14, 18). In the specialized cells of TAL constituting the macula densa, NKCC2 mediates signaling at the juxtaglomerular apparatus (32, 33, 47). The fundamental role of NKCC2 in renal salt reabsorption has further been firmly established in the context of salt wasting disorders and NKCC2-deficient mice (37, 52).

Data on the intracellular regulation of NKCC2 are scarce (13). Amino-terminal phosphorylation at three threonine residues (15), mediated by kinases such as WNK3 (39), may play a major role herein. AVP, glucagon, several other hormones, paracrine factors, and osmolality have been shown to influence NaCl transport as well as trafficking and biosynthesis of NKCC2 (14, 24, 27, 34, 46). AVP is particularly effective in enhancing NKCC2 and salt transport via G\textsubscript{i-protein}-coupled V2 receptor (V2R), cAMP release, and PKA activation preferentially in medullary TAL (1, 14, 18, 29). Localization of NKCC2 in subapical vesicles suggested membrane translocation of the transporter in the acute response to AVP/cAMP (31). In accordance with this, acute AVP-dependent phosphorylation of NKCC2 was associated with vesicular trafficking of the transporter to the luminal membrane (14).

Membrane proteins can be organized in microdomains (lipid raft, LR). LR are liquid-ordered phase, discrete domains enriched in glycosphingolipids and cholesterol. The raft hypothesis proposes that phase separation causes these lipids and proteins to aggregate in LR, determining their insolubility in nonionic detergents (for review, see Refs. 4, 36, 50). In the biosynthetic pathway, proteins may enter LR at the Golgi level and form complexes with other membrane proteins. Their shuttling between the Golgi and the cell membrane is probably a way for the cell to exert regulatory control over the surface expression of the proteins (30, 51). Lipid-binding proteins such as caveolins or flotillins may help to organize LR (9, 44). Although there has been controversy about proper definitions in the raft concept, there is now little doubt about their functional involvement in polar sorting, turnover, and signaling properties of membrane proteins (4, 22, 48).

Meanwhile, it has been shown that apart from hydrophobicity, other factors such as transmembrane domain amino acid sequence, membrane-proximal cytoplasmic or extracellular motifs, or components of adhering macromolecular complexes may affect raftophilicity (2, 4, 6, 11, 38, 53). Accordingly, there is now growing literature to demonstrate that membrane-multispanning ion channels and transporters such as the Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 (NHE3), Na\textsuperscript{+}/Pi cotransporter-2 (NaPi-IIa), epithelial Na\textsuperscript{+} channel (ENaC), and basolateral Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (NKA) may distribute in LR (20, 21, 28, 55).

In this study we hypothesized whether surface expression, activity, and vesicular trafficking of NKCC2 depend on its distribution in LR. We have studied a functional role for the lipid environment in NKCC2-dependent ion transport using an

* P. Welker and A. Böhlick contributed equally to this work.

Address for reprint requests and other correspondence: S. Bachmann, Institute of Anatomy, Charité-Universitätsmedizin Berlin, Philippstr. 12, 10115 Berlin, Germany (e-mail: sbachm@charite.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
EXPERIMENTAL PROCEDURES

Animals. Adult male Sprague-Dawley (SD), Long Evans (LE), and Brattleboro rats with hereditary hypothyroidic diabetes insipidus (DI) weighing between 200 and 250 g were used. LE and DI rats had been obtained from Harlan (Indianapolis IN) for breeding. All rats were bred in the local animal facility under conditions agreed to by the German law for animal protection (Berliner registra no. G357/05). Homozygous DI and control LE rats received treatments to induce trafficking of NKCC2; the selective V2 vasopressin receptor agonist 1-deamino-8-b-arginine vasopressin (dDAVP; 1 μg/kg body wt) or vehicle (0.9% NaCl) were given to both strains by intraperitoneal injections (4 groups, total n = 20 rats). To obtain kidneys for cell isolation or biochemical analysis, rats were isoﬂurane anesthetized and kidneys were removed via opening of the abdominal cavity 1 h after drug application. For histochemical evaluation of kidneys, rats were given an intraperitoneal injection of Nembrutal (40 mg/kg body wt) and were aldehyde-ﬁxed by retrograde perfusion through the abdominal aorta (46). Kidneys were then removed and prepared for cryostat sectioning or parafﬁn embedding. For immunoelectron microscopy, samples were postﬁxed in 3% paraformaldehyde (PFA) and 0.05% glutaraldehyde and then embedded in LR-White resin.

Cells. Cultured SV40-transformed rabbit TAL cells (rBTAL) were obtained from rabbit kidney medulla. Cells were seeded on culture dishes or glass coverslips and cultured in DMEM/Ham’s F-12 medium containing l-glutamine, 15 mM HEPES, 1% penicillin/streptomycin, 5% fetal calf serum, 1% l-glutamine, and 1% nonessential amino acids (GIBCO; 7.5% CO2, 37°C). Cells were utilized up to the 10th passage (55). For immunohistochemistry, cells were seeded in multwell tissue culture plates into which either coverslips coated with aminosilane (Sigma) or, alternatively, membrane inserts with a pore size of 0.4 μm (Falcon) had been placed. Cells were grown to conﬂuent monolayers and incubated with 7.5% CO2 at 37°C. Culture medium was changed every 2 days. Cell viability was tested using trypan blue (Sigma).

AVP treatment. For AVP treatment, cells were incubated in culture ﬂasks or on coverslips with 0.1 μM AVP (Sigma) in culture medium for 15, 30, or 60 min and 4, 6, or 24 h, harvested, and prepared for Western blot or immunostaining as detailed below. To study responsiveness to the cAMP signaling pathway under AVP stimulation (34), rBTAL cells were incubated for 1 h with the cAMP agonist 8-bromodenosine 3’,5’-cyclic monophosphate (8-Br-cAMP; 10 nM; Biolog) or the PKA inhibitors H-89 (10 nM; Bio ﬂavin) or the Rp diastereomer of adenosine 3’,5’-cyclic monophosphorothioate (Rp-cAMPs; 20 nM; Biolog) in the presence or absence of AVP. Cells had been preincubated for 45 min with 0.5 mM 3-isobutyl-1-methyl xantine (Sigma) to inhibit the activity of cAMP phosphodiesterase. Cells were then washed with cold PBS, harvested, counted in a Neubauer chamber, frozen and thawed twice, and centrifuged at 800 g. cAMP was detected in the supernatants using an ELISA kit (R&D Systems).

Influence of AVP on NKCC2 translation was studied by preincubation of rBTAL cells with 0.1 mM cycloheximide (Sigma) for 4 h, followed by an incubation for 1 or 4 h with AVP (0.1 μM) in the continued presence of cycloheximide. Whole cell lysates were analyzed by Western blotting. To study the role of the cytoskeleton, the actin-depolymerizing agent cytochalasin-B (20 μM; Sigma) was applied in the presence or absence of AVP.

Functional expression of NKCC2 in Xenopus laevis oocytes. Functional expression of NKCC2 was assessed as previously described (27, 35). In brief, defolliculated stage V–VI Xenopus laevis oocytes were injected with water or NKCC2 cRNA at 0.2 μg/μl and incubated for 3 days in frog Ringer ND96 containing sodium pyruvate and gentamicin. The next day, oocytes were exposed to 30 min of incubation in K+– and Cl–-free ND96 medium supplemented with 1 mM ouabain, followed by a 60-min uptake period in ND96 with 1 mM ouabain and 2.0 μCl of 86Rb+. Because X. laevis oocytes express an endogenous Na+–K+–2Cl– cotransporter (12), the mean value observed in water-injected oocytes was subtracted from the uptake observed in NKCC2-injected oocytes from each experiment. 86Rb+ uptake was assessed in the absence or presence of bumetanide. Values observed in parallel groups of oocytes injected with water were subtracted from values in corresponding groups of oocytes injected with NKCC2 cRNA to deﬁne the 86Rb+ uptake induced by NKCC2. At the end of the uptake period, oocytes were washed ﬁve times in ice-cold uptake solution without isotope, dissolved in 10% sodium dodecyl sulfate, and counted by beta scintillation counting. Each experiment was performed in duplicate.

Depletion of membrane cholesterol. For cholesterol depletion (CD), rBTAL cells were cultured in the presence or absence of 4 μM lovastatin and 0.25 mM mevalonate for 24 h and subsequently for 1 h with 10 mM methyl-β-cyclodextrin (MβCD; all from Sigma). To conﬁrm CD cytochemically, coverslips or membrane inserts were incubated for 15 min with green ﬂuorescent ﬁlhin (125 μg/ml in PBS at room temperature; Sigma), followed by nuclear staining with 4,6-diamidino-2-phenylindole (DAPI; dilution 1:10,000; Abcam). Coverslips or inserts were placed on microscopic slides in the presence of antifading ﬂuorescence mounting medium (DAKO). Oocytes were exposed to 10 mM MβCD alone for a total of 90 or 180 min, including the uptake period of 60 min. The use of MβCD to induce CD in X. laevis oocytes has been previously validated (45).

In situ hybridization. In situ hybridization was performed on perfusion-ﬁxed, paraﬁn-embedded tissue as described previously (29). Digoxigenin (DIG)-11-UTP-labeled antisense V2R riboprobes were hybridized and recognized with sheep anti-DIG-alkaline phosphatase-conjugated antibody (DAKO) diluted 1:50 in blocking medium. Signal was generated using 4-nitroblue tetrazolium chloride. Sections were analyzed in bright-ﬁeld microscopy.

Antisera. Antibodies used for NKCC2 in this study were guinea pig antirabbit NKCC2 antibody (2.1; 1:250 dilution) (29, 46), rabbit antirat NKCC2 antibody (1:250 dilution; Biotrend), and mouse monoclonal anti-rabbit NKCC1/2 antibody (T4; 1:250 dilution; Developmental Studies Hybridoma Bank). Other antibodies used in this study were rabbit anti-human THP (1:200 dilution) (46), mouse monoclonal anti-ﬂotillin-1 antibody (1:500 dilution; BD Biosciences), mouse monoclonal anti-caveolin-1 antibody (1:200 dilution; Santa Cruz Biotechnology), rabbit anti-human zona occludens (ZO)-1 antibody (1:500 dilution; Invitrogen), mouse monoclonal anti-clathrin antibody (1:200 dilution; Progenie), rabbit polyclonal anti-vasopressin receptor (V2R) antibody (1:300 dilution) (29), and rabbit polyclonal anti-ganglioside asialoGM1 (1:500 dilution, Abcam). For double staining, speciﬁc sera were combined with FITC-conjugated phallolidin, staining actin ﬁlaments (Molecular Probes). Secondary antibodies were coupled to horseradish peroxidase (HRP; DAKO), Cy3, or Cy2 (both from Dianova).

Immunohistochemical analysis. Crystalline CD sections (7 μm) or paraffin sections (4 μm) were prepared for immunohistochemical study of kidneys. For immunoelectron microscopy, ultrathin LR-White sections were prepared on grids. To analyze cultured cells, these were grown on coverslips and ﬁxed with cold methanol or, alternatively, 3% PFA in PBS. In brief, sections and cells were blocked with 5% milk powder dissolved in PBS and incubated with speciﬁc antibodies dissolved in the blocking solution overnight at 4°C; after thorough rinsing in PBS, appropriate secondary antibodies were applied at room temperature (55). Monolayers from primary cell culture were selected for evaluation when a proportion of 20–30% of the cells was NKCC2 immunoreactive. Immunoelectron microscopy staining was performed on grids, and signal was detected with 10-nm IgG-coupled
immunogold (Auroprobe). All antibodies used for immunohistochemistry had been previously characterized elsewhere; specificity was therefore controlled only by omitting the first antibody.

Conventional fluorescence microscopy and quantitative confocal laser scanning microscopy. Conventional fluorescence microscopy was performed in a DMRB microscope (Leica) equipped with a digital camera (Spot 32; Diagnostic Instruments); images were digitized using Metaview software (Visitron). For confocal microscopy and quantitative evaluation of the cell culture experiments, a confocal laser scanning microscope equipped with a multilaser system (argon laser, 458–514 nm; helium-neon laser, 543 nm; and helium-neon laser, 633 nm; Leica) and a Leica confocal software package was used. The observer was blinded to the treatment of the individual samples. From each experiment, five slides per individual condition, cytochemically labeled by single or double staining, were analyzed by randomly selecting five optical fields per slide. Multichannel detection was checked by sequential scanning analysis to avoid overlapping of fluorescence emission signals in double-stained conditions. Per field, the sum of pixel intensities in the selected stacks of interest of the apical vs. basolateral cell compartments were evaluated. The dynamic range was set such that sites with the most intensive fluorescence had only a few saturated pixels. Fluorescence signal intensities were quantified under standard digital scanning conditions with the basic microscope settings (laser intensity, photomultiplier tube offset, and gain) adjusted uniformly for the evaluation of the individual samples.

Cholera toxin-NKCC2 double staining. To study potential colocalization of NKCC2 with the LR component ganglioside GM1, primary TAL cells and rbTAL cells grown on coverslips were incubated with a cholera toxin-B chain (CT-B)-Alexa 594 conjugate (10 μg/ml) that binds to GM1, followed by incubation with antibody to CT-B; the conjugates were then cross-linked with PFA according to the manufacturer’s instructions (Vybrant lipid raft labeling kit; Sigma). Next, the cells were incubated with anti-NKCC2 (2:1) for double staining, followed by staining with Cy3-coupled secondary antibody.

Tissue and cell homogenization and fractionation. Tissue blocks of kidney inner stripe or cortex were frozen in liquid nitrogen, ground in a mortar, and thawed in buffer I (250 mM sucrose, 10 mM triethanolaminate, protease inhibitors (Complete; Roche Diagnostics), pH 7.5) (55); alternatively, 500 mM sodium carbonate, 5 mM dithiothreitol, and the protease inhibitors were used at pH 11. rbTAL cells grown to confluence were harvested in ice-cold PBS with a plastic scraper (20 petri dishes per experiment, diameter 9 cm; Falcon) and pelleted. The pellet was frozen in liquid nitrogen and then thawed in buffer I. Tissues and cells were subsequently lysed by ultrasonication (5 × 5 s with 4°C cooling intervals) to produce extracts. These were centrifuged at 300 g (10 min, 4°C) to remove nuclei and whole cells or, alternatively, at 4,000 g (15 min, 4°C) to also remove mitochondria (P1). For preparation of membrane fractions, the resulting postnuclear supernatant (S1) was centrifuged at 18,000 g (60 min, 4°C) to obtain a pellet containing plasma membrane and adherent vesicular structures (P2) and a supernatant (S2) containing the intracellular fraction with cytoplasmic and vesicular components.

Detergent extraction using Triton X-100. The postnuclear supernatant (S1) was centrifuged at 120,000 g (60 min, 4°C) to concentrate the plasma membrane and cytoplasmic vesicular structures. The pellet was homogenized in buffer I with a 26G syringe and incubated with ice-cold Triton X-100 (final concentration 1%, 60 min, 4°C). Alternatives to Triton X-100 (Triton X-114, Brij-98, and CHAPS) have been tested as well.

Sucrose gradient fractionation (floating assay). Triton X-100-treated cell or kidney homogenates were resuspended in buffer I and processed using two alternative floatation techniques that differed with respect to gradient composition (55). In brief, the detergent-insoluble fraction was resuspended in 1 ml of buffer I containing 40% sucrose, overlayed with 2 ml of 30% sucrose plus 2 ml of 5% sucrose in buffer I (discontinuous floating assay). Both gradients were then centrifuged at 200,000 g (16 h, 4°C). From the discontinuous gradients, 3 fractions (1 ml of 40%, 2 ml of 30%, and 2 ml of 5% sucrose) were used for overview study and parallel lipid analysis or 10 fractions (200 μl each) for high-resolution analysis were sequentially collected from the top of the gradients and analyzed using gel electrophoresis and Western blotting. For control purpose, cell or kidney homogenates were incubated with Triton X-100 (1%) at 37°C for 1 h to study disintegration of LR.

Lipid analysis. Fractions of floating assays derived from rat kidney outer medullary membrane preparations were resuspended in 50 mM Tris-HCl, 150 mM NaCl, 0.5% Na-deoxycholate, and 20% methanol (pH 7.4), blotted onto nitrocellulose, verified by Ponceau red, and analyzed by dot-blot staining for GM1 using specific antibody and peroxidase detection. For further lipid detection, extracts were resuspended in 100 μl of 50 mM HEPES adjusted to pH 8.0 in HEPES and mixed with 200 μl of methanol-chloroform (1:1). After Bligh-Dyer two-phase extraction, lipids were separated by thin-layer chromatography (TLC) using a mixture of chloroform-methanol-H2O (65:25:4). Lipids were visualized using 20% H2SO4, at 120°C. Cholesterol, phosphatidylcholine, and sphingomyelin were used as standards (all from Sigma). Dot blot and TLC signals were evaluated densitometrically.

Ultrastructural analysis of LR. LR fractions from floating assays with membrane preparations from rat kidney outer medulla were resuspended in HEPES (pH 7.3) and prepared for fixation or Western blot control using anti-flotillin-1 antibody. Pooled pellets were fixed in 3% PFA and 0.05% glutaraldehyde and embedded in LR-White resin. Immunogold labeling on the grids was performed with anti-NKCC2 antibody (T4) and 10-nm size immunogold for detection as described above. Ultrathin sections were examined in a Zeiss EM 12.

Surface biotinylation. Control rbTAL cells as well as cholesterol-depleted and AVP-treated rbTAL cells were prepared as detailed above. Cells were grown in culture flasks and washed with ice-cold PBS (pH 7.5) for surface biotinylation using sulfo-NHS-biotin (Pierce) according to the manufacturer’s instructions. In brief, cells were incubated for 30 min with the biotin reagent, followed by removal of the solution and quenching of remaining reagent with PBS containing 100 mM glycine. Cells were then scraped, washed with cold PBS, and centrifuged to produce a postnuclear supernatant (S1) that was further centrifuged at 18,000 g for 30 min to obtain the membrane fraction (P2) and a supernatant (S2; cytoplasmic and vesicular fraction). P2 and S2 fractions were incubated with protein G-coupled microbeads for magnetic cell separation (MACs, Miltenyi) according to the manufacturer’s instructions. The protein G-coupled beads were incubated with rabbit anti-NKCC2 antibody (1:200 dilution) and mixed with the P2 and S2 fractions for 1 h on a rotating platform at 4°C. Beads were then bound to columns placed in a magnetic field, and
columns were washed by sucrose buffer three times to remove unbound proteins. Immunoprecipitated protein was then eluted from the columns. Eluates were analyzed by Western blot. To this end, beads were run on 8% SDS-PAGE, blotted onto nitrocellulose, and detected with streptavidin-coupled HRP (1:5,000 dilution; DAKO) or guinea pig anti-NKCC2 (1:250 dilution); signal was generated by anti-guinea pig antibody coupled to HRP (1:4,000 dilution; DAKO).

RT-PCR. Total RNA was isolated from whole kidney homogenates or cell lysates using the RNeasy total RNA kit (Qiagen). To perform RT-PCR, genomic DNA was digested by DNase. cDNA was synthesized by reverse transcription of 5 μg of total RNA using a cDNA synthesis kit (Invitrogen). For amplification of V2 vasopressin receptor, 5′-TgT ggC TCT gTT TCA AgT gC-3′ forward primer and 5′-gTg CCA CAA ACA CCA TCA Ag-3′ reverse primers were used. For amplification of NKCC2, 5′-ggC ACT ggg AgC ATg AAT gAC-3′ forward primer and 5′-AAAA CCC TgA CAC CAT gCT CAT-3′ reverse primer were used. For specific detection of three alternatively spliced NKCC2 cassette exons (33), 5′-gTC TTg gAg TtC CAC gAA CAA ACC CgT TA-3′ reverse primer for the A-isoform, 5′-gTC TTg TgT TgA TTA TCA TC-3′ forward primer and 5′-gTC CTC TgA CAT ATC CAT TT-3′ reverse primer for the B-isoform, and 5′-ATC gTC ATT ggC Ctg AgT gtT-3′ forward primer and 5′-gTC CTC TgA CTA CTC CAT TT-3′ reverse primer for the F-isoform were used. RT-PCR reactions were carried out in an automated thermal cycler (Perkin Elmer, Boston, MA) using Taq polymerase (GIBCO). Reactions were controlled by PCR amplification of the housekeeping gene β-actin. Quantitative gene expression studies were performed using TaqMan gene expression assays for NKCC1 and NKCC2 (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems). GAPDH expression was used as an endogenous control.

Presentation of data and statistical analyses. Results are means ± SD. Statistical significance was determined using Student’s unpaired t-test. P < 0.05 was considered significant.

RESULTS

Rat kidney and cultured TAL cells specifically express vasopressin receptor along with NKCC2. To study the effect of AVP on NKCC2 in association with LR, we have confirmed histochemically the key gene products expressed in the studied tissue and cells. NKCC2 immunostaining and concomitant V2R mRNA expression have been demonstrated in rat outer medullary tissue and cells. NKCC2 immunostaining and concomitant V2R histochemically the key gene products expressed in the studied systems. GAPDH expression was used as an endogenous control.

Results are means ± SD. Statistical significance was determined using Student’s unpaired t-test. P < 0.05 was considered significant.

Fig. 1. Cytochemistry and RT-PCR characterize rabbit cells of the thick ascending limb of the loop of Henle (rbTAL) cell monolayers as used in lipid raft (LR) studies. A–C: conventional immunofluorescence shows Na⁺/K⁺-Cl⁻ cotransporter 2 (NKCC2; red) in a widespread distribution across the cell; cell borders are zonula occludens (ZO)-1-positive (green), nuclei are 4,6-diamidino-2-phenylindole (DAPI)-stained (blue). D–F: preincubation peptide blockade (10-fold excess) of the antibody against NKCC2 reveals near-total absence of specific signal. G and H: RT-PCR showing the presence of mRNA coding for NKCC2 and its 3 isoforms (F, B, and A) with specific, distinct primers and of vasopressin receptor (V2R) mRNA. I and J: real-time PCR assay showing representative expression of NKCC2 (J) vs. NKCC1 mRNA (I) in rabbit kidney (I), rbTAL cells (2), and rat kidney extracts (3) from 4 independent experiments. Data are normalized for GAPDH mRNA, which was run in the same well, and are expressed as the change in cycle threshold (ΔCt).
There is a pool of NKCC2 in detergent-resistant, cholesterol- and sphingolipid-enriched membranes in rat kidney and cultured TAL cells. Major criteria to establish the association of proteins with LR are used to demonstrate that the protein is insoluble in cold detergent and that it shifts from lighter to heavier membrane fractions after CD based on density gradient fractionation (50). Detergent solubilization of membrane extracts from rat kidney and rbTAL cell homogenates and subsequent centrifugation resulted in the separation of detergent-soluble and -insoluble membranes. Lipid composition analysis by dot blot for GM1 and by TLC for cholesterol, phosphatidylcholine, and sphingomyelin re-

Fig. 2. A pool of membrane NKCC2 and related proteins from rat kidney is associated with LR-specific lipids. Purified membrane preparations from rat kidney extracts were solubilized with cold 1% Triton X-100 and assayed in sucrose gradient floating assays. Fractions show distribution of GM1 by dot blot (A), cholesterol, phosphatidylcholine, and sphingomyelin by TLC (B), and NKCC2 (C), co-localized THP (D), V2R (E), and Na+/Cl− cotransporter (NCC) (F) by Western blots in the low-density LR raft fractions. The reference protein flotillin distributes in rafts (G), whereas clathrin is typically absent from LR (H). Densitometric scanning analysis of blots and TLC bands from at least 4 independent experiments is shown above the respective representative samples for the gradients. Products are enriched in the LR fractions ranging near 20% sucrose. The bands for NKCC2 were located at 160 kDa, THP at 98 kDa, V2R at 50 kDa, NCC at 165 kDa, flotillin at 48 kDa, and clathrin at 180 kDa.
vealed enrichment of these lipids in the low-density fractions from rat kidney extracts separated by floating assay (Fig. 2, A and B). Plots indicate a heterogeneous distribution of these components along the different fractions. Western blots showed that 40–70% of NKCC2 was associated with the Triton X-100-insoluble fractions and the other 60–30% with the Triton X-100-soluble fractions at 160 kDa (Fig. 2C). Among different nonionic detergents tested for NKCC2’s resistance to solubilization, Triton X-100 produced the most marked NKCC2 insolubility. Reacting homogenates with warm (37°C) instead of cold Triton X-100 resulted in LR disruption and absence of NKCC2 and flotillin from the low-density fractions. The GPI-anchored glycoprotein THP, which is coexpressed with NKCC2 in the TAL, fractionated in parallel (Fig. 2D). The V2 receptor and the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter NCC, a second major member of the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters that is located in the downstream nephron segment ensuing TAL, fractionated in the buoyant membranes as well (Fig. 2, E and F). Flotillin and clathrin served as positive and negative controls, respectively (Fig. 2, G and H).

**Identification of NKCC2 by LC-MS/MS in LR fractions.** To corroborate Western blot identification of NKCC2 in LR, a high-specificity proteomic approach was performed using LC-MS/MS from rat kidney medullary LR fractions. The results demonstrated highly significant occurrence of a 19-amino acid LC-MS/MS from purified rat outer medulla revealed vesicles TM12 domain (see Supplemental Material 1).

**Ultrastructural analysis of LR membranes.** LR membranes prepared from purified rat outer medulla revealed vesicles 100–200 nm in diameter and membrane fragments. Immunogold staining with T4 antibody against NKCC2 showed labeling over some but not all of the membranes, reflecting that some but not all LR were from TAL (Fig. 3). The V2 receptor and the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter NCC, a second major member of the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters that is located in the downstream nephron segment ensuing TAL, fractionated in the buoyant membranes as well (Fig. 2, E and F). Flotillin and clathrin served as positive and negative controls, respectively (Fig. 2, G and H).

**Depletion of cholesterol reduces the pool of NKCC2 in LR.** To evaluate the effect of CD in rbTAL monolayers, cells had been treated with mevalonate-lovastatin and MβCD; absence of cytochemical staining for filamentous actin confirmed CD in the treated cells as described (55). Trypan blue staining of the cholesterol-depleted cells confirmed their viability, since no intracellular penetration of the dye was registered. CD caused a significant shift in the distribution of NKCC2 and flotillin from the low- to the high-density fractions, indicating partial solubilization of NKCC2 and the control protein (Fig. 4, A–C). The detection of clathrin in the high-density fraction was not influenced by this treatment.

**Depletion of cholesterol reduces NKCC2 activity in Xenopus laevis oocytes.** Oocytes were exposed to MβCD for a total of 90 or 180 min, including the uptake period of 60 min. The activity of NKCC2 was significantly reduced by CD in a time-dependent fashion (Fig. 4, D and E). At 90 min, the observed reduction was from 4,676 ± 313 pmol-oocyte\(^{-1}\)h\(^{-1}\) in the absence of MβCD to 3,121 ± 258 pmol-oocyte\(^{-1}\)h\(^{-1}\) in its presence (P < 0.05; Fig. 4D). At 180 min of exposure, the reduction was more pronounced. NKCC2-induced \(^{36}\)Rb\(^{+}\) uptake was reduced from 5,859 ± 570 pmol-oocyte\(^{-1}\)h\(^{-1}\) in the absence of MβCD to 802 ± 109 pmol-oocyte\(^{-1}\)h\(^{-1}\) in its presence (P < 0.01; Fig. 4D). The observed reduction with these times of incubation represented 33 and 87% NKCC2 inhibition, respectively (Fig. 4E). These observations strongly suggest a functional background for an association between NKCC2 expressed in oocytes and their plasma membrane cholesterol content.

**Effect of short-term AVP administration on apical trafficking and the association of NKCC2 with LR.** To study whether V2R-mediated polar delivery of NKCC2 is related to cholesterol-dependent, LR-associated trafficking upon AVP stimulation, we performed experiments in vivo, in cultured rbTAL monolayers, and in primary TAL cell culture. V2R mRNA was strongly expressed in medullary TAL profiles of LE and DI rats (Fig. 5, A and B). In LE rats, and more drastically in Brattleboro DI rats, treatment with AVP (intraperitoneal injection of a bolus of 1 μg/kg, 1 h) showed significant changes in apical NKCC2 abundance compared with the respective controls receiving vehicle injection (Fig. 5, C and D). In rbTAL cells, an AVP-induced shift of immunoreactive NKCC2 toward the luminal membrane was observed along with a general increase in apical signal intensity for NKCC2 (Fig. 5, E and F; vehicle vs. 10\(^{-7}\) M AVP, 1 h). In vivo, NKCC2 also fractionated to a significantly increased proportion (almost 2-fold) into the low-density range of the plasma membrane fractions as used throughout for the floating assays; this was independent of the general increase in NKCC2 signals upon AVP (Fig. 6A). Flotillin was unaffected under this condition (Fig. 6B). Analogous observations were made in cultured rbTAL cells (see Supplemental Material 2). In these cells, the AVP-dependent shift from a juxtanuclear position of immunoreactive NKCC2 toward an apical concentration was further specified using confocal series of stacks (Fig. 7A). A sharp increase in mean apical fluorescence intensity was detectable as early as after 15 min, followed by further augmentation after 4 h. Parallel evaluation by Western blot showed a moderate increase in NKCC2 abundance as early as after 1 h and a more marked increase after 4 h of AVP treatment, whereas after 24 h, signal was decreased to near control level (Fig. 7B). Cycloheximide, an inhibitor of protein translation, did not prevent the rise in NKCC2 signal after

![Fig. 3. Ultrastructural immunogold labeling of LR isolated from rat kidney outer medulla shows NKCC2-positive membranes. LR were prepared from purified membrane preparations after solubilization with cold 1% Triton X-100 and sucrose density gradient centrifugation technique. The detergent generates vesicle-like and fragmentary LR ~200 nm in diameter. Rafts were incubated with antibodies against clathrin (A), NKCC2 (T4 antibody; B), THP (C), and GM1 (D). A subset of LR shows positive NKCC2 immunostaining. Bar, 200 nm.](image-url)
1 h of AVP treatment but blunted the increase after 4 h (Fig. 7C). Specificity of the AVP-induced changes was verified by incubating the cells with 8-Br-cAMP, leading to adluminal increase of NKCC2 fluorescence close to the level that was reached after AVP application (Fig. 7D). Further controls included the PKA inhibitors H-89 and Rp-cAMPS, both of which reduced AVP-dependent stimulation of NKCC2 significantly. Cytochalasin B markedly blunted the effect of AVP as well, suggesting that apical trafficking of NKCC2 depends on actin polymerization.

LR markers such as CT-B, staining ganglioside GM1, produce fluorescent patches in the cell, indicating LR sites. These may copatch with LR proteins, resulting in an overlapping fluorescence signal. Copatching was therefore used as a microscopy assay for raft association. After 1 h of AVP treatment, NKCC2 revealed a clear increase in copatching of the two signals in confocal imaging as visualized by the merge images; the individual signals not only for NKCC2 but also for CT-B were enhanced. The overlap is demonstrated semiquantitatively (Fig. 8). These findings were corroborated using rat primary cultured TAL cells from the inner stripe (see Supplemental Material 3). CD by combined mevalonate lovastatin and methyl-β-cycloheximide (MβCD) treatment to inhibit cholesterol synthesis and disrupt LR. Rafts were prepared from purified membrane preparations after solubilization with cold 1% Triton X-100. Floating assays demonstrated cholesterol dependence in the partitioning of NKCC2 (A) and flotillin (B), but not clathrin (C), in LR. Densitometric scanning analysis of Western blots from 5 independent experiments is shown above the respective, representative Western blots. CD caused significant rightward shifts of NKCC2 in the LR fractions ranging near 20% sucrose. *P < 0.05 compared with vehicle. D and E: NKCC2 activity in X. laevis oocytes is reduced by CD. Oocytes were injected with water or NKCC2 cRNA, and 86Rb+ uptake was assessed. D: NKCC2-dependent 86Rb+ uptake in the absence (vehicle) or presence of 10 mM MβCD during 90 or 180 min, as indicated. E: normalized NKCC2-dependent 86Rb+ uptake (after subtraction of uptake in water-injected oocytes) in the absence of MβCD set as 100%. *P < 0.05 compared with vehicle. Data in both D and E are pooled results from 2 independent experiments with 10 oocytes per group each.

Biotinylation assays show the amount of surface-expressed NKCC2 is increased upon short-term AVP stimulation of rbTAL cells in a cholesterol-dependent manner. We ultimately aimed to consolidate our hypothesis that AVP not only causes apical trafficking of NKCC2 but also promotes luminal insertion of a pool of NKCC2 in a cholesterol-dependent manner to modulate transepithelial NaCl transport via LR. To this end, the surface of live rbTAL cell monolayers was biotinylated. The monolayers were then homogenized and cell membrane and intracellular fractions prepared. Fractions were immunoprecipitated with anti-NKCC2 antibody. The abundance of biotinylated NKCC2, which was determined by streptavidin-peroxidase labeling, was compared with the total abundance of NKCC2, as determined using a different anti-NKCC2 antibody, in the respective fractions (Fig. 10). Samples receiving vehicle showed dramatic reductions in NKCC2 in the membrane fractions upon CD with either detection system (Fig. 10, A and B), whereas in the intracellular fractions, total NKCC2 was not significantly changed (Fig. 10, C and D). As expected, the amount of intracellular biotinylated NKCC2 was very low.
Evidence for partitioning of NKCC2 into cholesterol- and sphingolipid-enriched LR. Results of this study have extended the current concepts of cellular distribution, function, and regulated polar delivery of the major renal epithelial cotransporter NKCC2. Our first major point was to confirm the hypothesis that NKCC2 may distribute in LR with a functional background. As a biochemical hallmark for LR localization, we have applied the procedure of cold extraction with nonionic detergent to isolate LR, relying on their relative, differential insolubility in the detergent in sucrose gradient fractionation, which is based on the close packing of acyl chains and stabilization via cholesterol interdigitation (50).

We have shown that a pool of both intracellular and plasma membrane NKCC2 is LR associated based on the following observations. 1) Forty to seventy percent of NKCC2 obtained from the extracted plasma membrane preparations including adherent vesicular membranes was detected in the low-density range of the floating assays. The presence of the transporter in LR fractions was identified by Western blot and highly specific LC-MS/MS. 2) LR properties were confirmed by the typical enrichment in ganglioside GM1, sphingolipid, and cholesterol selectively in the low-density fractions (55). 3) NKCC2 was detected immunoelectron microscopically in a subset of membrane fragments obtained from sucrose fractionation, reflecting data on intestinal LR proteins (7). 4) Binding of CT-B to GM1-containing LR, a natural process adapted for experimental LR recognition (17), showed copatching of a subset of CT-B and NKCC2 within the cell and on the surface. 5) LR association of NKCC2 depended on cholesterol availability as a widely used criterion (19, 28, 55). Localization of NKCC2 in LR of the plasma membrane further agrees with its histochemical localization of NKCC2 in subapical and apical TAL epithelium (14, 31). The occurrence of LR carrying NKCC2 in intracellular fractions suggested their involvement in apical delivery of NKCC2, as described for other membrane-spanning proteins (19, 28). Parallel detection of the structurally related cotransporter NCC (13) in LR frac-

**DISCUSSION**

in the respective fractions (Fig. 10, C and D). Mirroring the histochemical results shown in Fig. 5, E and F, and Fig. 7A, Fig. 10, A and B, further shows pronounced increases for biotinylated NKCC2 (nearly 3-fold) and total NKCC2 in the cell membrane fraction after 1 h of AVP stimulation. Both effects were blunted by CD. These observations permit the conclusion that both steady-state as well as AVP-stimulated surface expressions of NKCC2 are markedly cholesterol dependent. This finding agrees with the concept that LR-dependent trafficking may be relevant for NKCC2 in its active, luminally inserted form. Corresponding to the increases in total cell NKCC2 shown in Fig. 7B, intracellular NKCC2 abundance showed a pronounced stimulation upon AVP; this increase was effectively blunted by CD (Fig. 10, C and D). In agreement with our results from the floating assays and the confocal analysis, the latter observation reflects intensified packing of NKCC2 in LR-containing intracellular membranes upon the stimulatory effect of AVP. Packing into LR thus appears to be linked to the cellular adjustment of NKCC2 abundance.

**Fig. 5.** NKCC2 surface expression of rat medullary TAL and cultured rbTAL cells increases upon treatment with vasopressin (AVP). V2R mRNA (A) and immunoreactive NKCC2 (B) distributed in untreated control Long Evans (LE) rat medullary TAL. Intraperitoneal bolus injections of vehicle (0.9% NaCl; C) or 1-deamino-8-D-arginine vasopressin (dDAVP; 1 µg/kg; D) in Brattleboro rats with diabetes insipidus (DI rats) after 1 h; adluminal immunoreactive NKCC2 signal is enhanced upon treatment with dDAVP. A, in situ hybridization; B–D, immunoperoxidase staining with anti-NKCC2. Comparable effects are shown in vehicle (E) and AVP (1 × 10⁻⁷ M; 1 h)–treated rbTAL cells (F). Confocal merge signals of NKCC2 (red) and phalloidin identifying intracellular F-actin (green) are shown in the Z-axis with the apical side oriented toward the asterisk. Significant augmentation of red fluorescent NKCC2 label along with a shift toward the apical cell pole (asterisk) are shown.

**Fig. 6.** AVP administration increases the pool of NKCC2 in LR from rat kidney outer medulla. An intraperitoneal bolus injection of vehicle (0.9% NaCl) compared with dDAVP (1 µg/kg) in DI rats after 1 h increased the proportion of NKCC2 (A) but not flotillin (B) partitioned in LR. Rafts were prepared from purified outer medullary membrane preparations after solubilization with cold 1% Triton X-100 and sucrose gradient flotation technique. Densitometric analysis of Western blots from 4 independent experiments with fractions F3–F6 were evaluated cumulatively. Representative Western blots are shown at bottom with the evaluated fractions boxed (discontinuous gradients). *P < 0.05 compared with vehicle.

*AJP-Renal Physiol • VOL 295 • SEPTEMBER 2008 • www.ajprenal.org*
tions from rat kidney further strengthened our view; apparently the two products are using similar posttranscriptional pathways during biosynthesis.

Protein markers for raft and non-raft domains confirm LR association of NKCC2. Among the positive reference proteins, flotillin-1 as a well-established LR marker (44) and THP as a GPI-anchored raft- and TAL-specific product (5) were detected in the buoyant membrane fractions along with NKCC2. Apart from demonstrating specificity of the assays, the presence of flotillin suggests a role to organize LR as specialized membrane domains interacting with the cytoskeleton (4). Another well-defined, intrinsic component of LR is caveolin, which may target other proteins to LR in certain cell types (9). However, caveolins are not solely responsible for LR association of proteins, and the known caveolin isoforms were not detected in TAL (3, 42). The coated pit-related protein clathrin was used as an established non-raft marker (20, 55).

Cholesterol depletion allows mechanistic insight into polar delivery of NKCC2 in TAL and ion transport function of NKCC2 in the oocyte system. We have administered cholesterol-depleting agents to study whether the liquid-ordered biophysical properties stabilizing LR may influence NKCC2 characteristics. MβCD, a cyclic oligosaccharide acting like an external sponge to remove membrane cholesterol by adhesion contact to the cell surface, was applied acutely to withdraw lipid from the plasma membrane and reduce LR under established conditions (28, 43, 45). To prevent LR association of proteins during their transit through the Golgi apparatus and polar trafficking (28, 51), CD was reached in a two-step approach using mevalonate-lovastatin during 24 h to inhibit de novo synthesis of cholesterol, added with a final short period of MβCD treatment. Cholesterol in the entire cell may thus be reduced to near 80% (10). Neither this measure nor MβCD alone should cause general deficits in membrane structure and function (28, 45, 55).

Fig. 7. Time-dependent effects of AVP stimulation on NKCC2 trafficking and protein abundance in rTAL cells. A: time-dependent effects of AVP on NKCC2 surface expression are shown by semiquantitative evaluation of apical NKCC2 fluorescent signal. Values represent mean fluorescence intensity from 3 pooled apical confocal stacks. Baseline apical fluorescence was set at 100%. Representative confocal images are shown at bottom. B: time-dependent effects of AVP on NKCC2 protein abundance are shown by semiquantitative evaluation of Western blots from whole cell lysates and densitometric scanning. Results are normalized for the housekeeping protein β-actin. Representative blots are shown at bottom. C: as in B, time-dependent effects of AVP (1 and 4 h) on NKCC2 abundance are shown in the absence vs. presence of cycloheximide (cyclo). D: specificity of the AVP-induced changes in NKCC2 surface expression was demonstrated by application of the cAMP analog 8-bromo-cAMP (8-Br-cAMP), AVP alone, the PKA inhibitors H-89 and Rp-cAMPS, and cytochalasin B (CyB), each combined with AVP. Values were obtained from confocal microscopy of apical NKCC2 immunofluorescent signal. Values represent mean fluorescence intensity from pooled apical confocal stacks. Results are from 3 (A), 4 (B), 5 (C), and 3 independent experiments (D), respectively. *P < 0.05 compared with vehicle. **P < 0.05 compared with AVP.
In the rbTAL model, Western blot analyses from the floating assays showed that two-step CD induced significant shifts of NKCC2 along with flotillin from lower to higher density fractions, reflecting similar data from elsewhere (20, 28, 55). The histochemical findings further elucidate that LR and their association with NKCC2 in plasma membrane as well as cytoplasmic vesicles were strongly reduced after CD. Studying *X. laevis* oocytes, which have been widely used by us and others as a robust heterologous expression system of rat NKCC2 (13, 15, 27, 39), we have obtained important functional insights into NKCC2 function in conjunction with cholesterol availability. The prominent, highly reproducible decreases in NKCC2-induced $^{86}\text{Rb}^+$ uptake upon CD by MβCD in a time course of up to 3 h were suggestive of a major role for the integrity of a cholesterol-enriched membrane environment in transporter functioning. Previous analyses of other LR-associated transporters have provided divergent results with respect to their lipid environment (21, 28); for instance, CD failed to affect transepithelial Na$^+$ channel activity or expression of ENaC (20, 49). However, CD-induced effects may plausibly diverge among transporters and channels, since the N(K)CC family of proteins differs substantially from ENaC subunits with respect to molecular structure and functional requirements (13, 20). Alterations in membrane fluidity may have affected NKCC2 activity in the oocytes upon MβCD, as shown for a cation-conducting acetylcholine receptor (45). Since the typical distribution of LR in the plasma membrane of TAL cells probably agrees with the original concept of integral proteins distributed in LR or in non-raft membrane domains (25, 50), an equilbrium of silent vs. functional pools of NKCC2 might be effectively adjusted by changes in lipid composition, depending on their LR association (45). Our oocyte results may well resemble the in vivo membrane condition with its particular LR characteristics, since raft properties also have been established in the oocyte cell membrane (26).

Trafficing and polar delivery of NKCC2 on AVP is LR dependent. Our second major point was to investigate the involvement of LR in vesicular trafficking and apical delivery of NKCC2. Short-term exposure to AVP, a potent stimulus not only for water channel and NKA activation in collecting duct (8) but also for transepithelial NaCl reabsorption across TAL (1, 18, 27, 55) was therefore chosen to test our hypothesis. Established parameters for the activation of NKCC2 by short-term AVP application, such as V2R signaling and the particular effects of cAMP, PKA, and intactness of the actin cytoskeleton, have been demonstrated successfully, proving viability of the rbTAL cell line in this respect (1, 18, 29, 54, 55).

Short-term AVP treatment in the present setting produced two per se fundamentally different effects. One was related to changes in NKCC2 abundance and the other to LR-associated trafficking of the transporter. We earlier showed that cellular NKCC2 abundances in Brattleboro DI rats lacking endogenous AVP and in rbTAL cells were increased nearly twofold as early as after 30 min (29), and the present 1-h exposures to AVP produced similar results. As discussed previously (29), these findings are at variance with previous data raised in mice in which comparable, moderately supraphysiological concentrations of AVP after 1 h had failed to produce changes in NKCC2 abundance (14); increases in NKCC2 protein abundance have formerly been reported only from prolonged treatment periods (23). However, DI rats in our hands served as a suitable model for an induction of NKCC2 unbiased by endogenous AVP levels (29), and comparative setups with control rats also produced similar, albeit less drastic, results (unpublished data). Corresponding increases in NKCC2 abundance in rbTAL cells were highly reproducible and were further con-

![Fig. 8. Short-term AVP administration increases copatching of NKCC2 with the LR component, ganglioside GM1, in rbTAL cells. Confocal analysis shows the effects of short-term vehicle vs. AVP administration ($1 \times 10^{-7} \text{M}; 1 \text{h}$) on the degree of coincident fluorescence of immunostained NKCC2 and the cholera toxin-B (CT-B)-labeled ganglioside GM1. Diagrams at bottom indicate the degree of merged signals between CT-B plotted on the X-axis and NKCC2 on the Y-axis, indicating copatching.](F798.png)
firmed by parallel histochemical evaluation. Changes in
NKCC2 mRNA were absent, which could be expected regard-
ing its delayed induction (29, 40). Accordingly, inhibition of
translation by cycloheximide was without effect on NKCC2
abundance after 1 h of AVP but blunted its effect after 4 h.
Therefore, the observed effects were obviously unrelated to de
novo protein biosynthesis. Conceivably, the V2R-cAMP-PKA
signal cascade at some point may interfere with NKCC2
stability or so far unrecognized protein degradative mecha-
nisms in TAL.

AVP-induced recruitment of NKCC2 to LR and traffick-
ing were cholesterol-dependent as shown by 1) shifting of
NKCC2 toward the lower density fractions from DI rat
kidney and rbTAL cell extracts, 2) enhanced copatching of
NKCC2 and CT-B in rbTAL and primary cultured TAL cells
along with apical delivery, and 3) increased surface bioti-
nylation of NKCC2. Short-term AVP treatment produced
more than twofold increases in both surface-expressed and
total plasma membrane NKCC2 in rbTAL cells. Changes
were dependent on cholesterol availability. The response
showed a quick onset with 15 min. For comparison, GPI-
anchored proteins typically also appear at the apical mem-
brane within 15 min of leaving the Golgi in Madin-Darby
canine kidney cells (48). Conversely, abundance of NKCC2
was significantly diminished upon CD chiefly in the apical
cell pole or plasma membrane, but not in intracellular
fractions, as depicted in Figs. 9 and 10. Along the same line,
decreased apical NKCC2-immunoreactive signals under CD
probably reflected disaggregation of LR and diminished
apical delivery, since the CT-B-positive sites were also
obviously reduced in parallel. These observations suggest
that LR substantially interfere not only with trafficking but
probably also with stability of NKCC2 at the apical cell
pole. The failure to enhance trafficking and luminal inser-
tion of the transporter upon AVP treatment in the absence of
LR underscores this interpretation, suggesting that the
cholesterol environment of the transporter molecules or the
structure of LR proper are required for the maintenance of a

Fig. 9. AVP-induced changes of NKCC2 in rbTAL cells are cholesterol-dependent. A: confocal analysis shows the effects of AVP (1 × 10^{-7} M; 1 h) with and
without CD by combined mevalonate-lovastatin (M/L)/MβCD treatment on NKCC2 surface expression. Values indicate quantification of the apical fluorescence
intensity from 3 pooled apical confocal stacks with the vehicle control set at 100%. Representative confocal images are shown at bottom. B: representative
Western blots from rbTAL cell extracts show specific bands for NKCC2 and densitometric evaluation with intensity values normalized for β-actin. C: ELISA
measurements of cAMP accumulation in rbTAL cells incubated for 1 h with vehicle or AVP, combined with CD induced by M/L, MβCD, or a combination of
M/L and MβCD. Results are from 3 (A), 4 (B), and 4 independent experiments (C), respectively. *P < 0.05 compared with vehicle.
sufficiently large apical pool of NKCC2 and appropriate trafficking to the plasma membrane. This interpretation is further in line with results on intestinal NHE3 (28) and renal ENaC (49), highlighting a role for LR specifically in apical trafficking of these proteins.

The effects of CD on AVP-dependent increases in NKCC2 abundance and translocation might be questioned regarding the obvious distribution of a pool of V2R itself in LR. V2R themselves could therefore be affected by the CD protocol, possibly resulting in changes in binding as shown for another receptor (41) or in downstream signaling effects. Monitoring intracellular cAMP levels, we accordingly found that AVP application under various conditions of CD caused diminished cAMP accumulation in rbTAL cells; however, albeit reduced, the AVP-induced changes were still significant, thus validating our observations.

Collectively, our results from rat tissue, *X. laevis* oocytes, and TAL cell culture experiments support the hypothesis that NKCC2 distributes in LR both intracellularly and on the cell surface and that cholesterol availability is required for its activity, AVP-induced trafficking, and delivery to the cell membrane. In the plane of the luminal membrane, LR could act as platforms that modulate NKCC2 activity by accumulating active pools of the transporter. We therefore suggest that LR are an essential component in NKCC2 biology and therefore play an important role in the function of renal volume regulation. New aspects for studying lipid effects on ion transporter function have thus been presented. Future investigation must define the nature of protein-lipid interactions of NKCC2 and clarify how LR may determine trafficking, function, and turnover of NKCC2 in mammalian TAL.

ACKNOWLEDGMENTS

We acknowledge the methodological help of J.-H. Frühau, M. Fromm, and D. Günzel; the gifts of rbTAL cells by R. Kinne (Bochum), NCC antibody by D. H. Ellison, and V2R antibody by W. Müller-Esterl; and the skillful technical assistance of F. Serowka, K. Riskowsky, and P. Landmann.

GRANTS

This study was supported by Deutsche Forschungsgemeinschaft Grant DFG-FOR667 (to S. Bachmann), National Institute of Diabetes and Digestive
REFERENCES


