Characterization of a long-term rat mTAL cell line

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Eng B, Mukhopadhyay S, Vio CP, Pedraza PL, Hao S, Battula S, Sehgal PB, McGiff JC, Ferreri NR. Characterization of a long-term rat mTAL cell line. Am J Physiol Renal Physiol 293: F1413–F1422, 2007. First published August 1, 2007; doi:10.1152/ajprenal.00426.2006.—A medullary thick ascending limb (mTAL) cell line, termed raTAL, has been established from freshly isolated rat mTAL tubules and cultured continuously for up to 75 passages; it retains characteristics of mTAL cells even after retrieval from storage in liquid nitrogen for several months. The cells express Tamm-Horsfall glycoprotein (THP), a TAL-specific marker, grow to confluence, exhibit a polygonal morphology characteristic of epithelial cells, and form “domes.” Detection of THP, Na⁺⁻K⁺⁻Cl⁻ cotransporter (NKCC2), Na⁺⁻K⁺⁻ATPase, and renal outer medullary K⁺ channel (ROMK) was achieved using indirect immunofluorescence and confocal microscopy. Western blot analysis of NKCC2 expression using two different antibodies revealed a band of ~160 kDa, and RT-PCR analysis demonstrated the presence of NKCC2 isoforms A and F, which was confirmed by DNA sequencing; transport of Cl⁻ into raTAL cells was inhibited by furosemide. Oubain- and bumetanide-sensitive oxygen consumption, an index of ion trans

The mTAL reabsorbs ~25% of filtered NaCl and is the site of action of “loop” diuretics. This water-impermeable nephron segment dilutes the tubular fluid and generates a hypertonic interstitium because it is able to reabsorb NaCl without reabsorption of water (5). The Na⁺ pump (Na⁺⁻K⁺⁻ATPase) on the basolateral membrane of the mTAL contributes importantly to this process. Reabsorption from the tubular fluid of Na⁺, K⁺, and Cl⁻ via the Na⁺⁻K⁺⁻2Cl⁻ cotransporter (NKCC2) on the apical membrane is linked to the recycling of K⁺ back to the tubular fluid via apical K⁺ (ROMK) channels (43). K⁺ recycling and movement of Cl⁻ from the apical to the basolateral side of the cell establishes a lumen-positive electrical potential that provides the driving force for reabsorption of Ca²⁺ and Mg²⁺ via a paracellular pathway (20).

Several approaches have been used to isolate mTAL cells, including immunodissection (1), growth of cultures from explants of microdissected TAL segments (4), centrifugal elutriation (10), and enzymatic dissociation of outer medullary tissue with subsequent density gradient separation (13). The latter technique was modified by Trinh-Trang-Tan et al. (36) and further modified in our laboratory (12) to establish mTAL cells in primary culture with a purity of ~90–95%. The latter technique, which can be performed in ~3 h, takes advantage of the anatomical arrangement in the inner stripe of the outer medulla; careful dissection of this region yields renal tissue that is more than 70% mTAL tubules and is used as a starting point for further purification. The inherent resistance to enzymatic degradation of the mTAL compared with potential contaminating cell types in the inner stripe permits a size exclusion step, which is performed after the enzymatic digestion step, to yield a highly purified tubule suspension that is placed in culture and yields mTAL cells in primary culture.

Our laboratory has used primary cultures of mTAL cells to evaluate eicosanoid-dependent mechanisms that affect ion transport pathways and most recently has addressed the role of tumor necrosis factor-α, which is increased by activation of the Ca²⁺-sensing receptor to regulate a cyclooxygenase-2-dependent mechanism that may contribute to salt and water homeostasis (11, 12, 14–16, 41, 42), and also has studied the stimulatory effect of bradykinin on cyclooxygenase-2 (32). Whereas cells from the mTAL grow readily in primary culture, they are relatively difficult to prepare and resist passage. In the present study, we describe the characteristics of a long-term rat mTAL cell line, termed raTAL, which does not dedifferentiate and retains salient features of mTAL cells. Using immunofluorescence and confocal microscopy, we identified and localized the characteristic markers of these cells. The cells can be

The study of cellular mechanisms in the kidney is complicated by the heterogeneity of cell types along the nephron. Accordingly, several laboratories have developed approaches to study cell types isolated from discrete nephron segments. Compared with the large number of studies conducted in proximal tubules, far fewer studies have been performed in the medullary segment of the thick ascending limb (mTAL), primarily because of the difficulty in obtaining sufficient numbers of cells in high purity (33). In some instances, the use of isolated cells from different nephron segments is an attractive alternative to studies using renal slices, isolated tubules, or intact kidneys. However, the use of primary cell cultures has several limitations, including the relatively small number of cells available for study and their limited life span in culture.

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cultured continuously for more than 1 yr and grown in culture following long-term storage in liquid nitrogen. This cell line may be a useful model for evaluating biochemical, physiological, and molecular interactions, including trafficking of transporter molecules in the mTAL.

**METHODS**

**Animals**

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 100–110 g were maintained on standard rat chow (Ralston-Purina, Chicago, IL) and given tap water ad libitum. All protocols were in accord with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received prior approval by the Institutional Animal Care and Use Committee at New York Medical College.

**Reagents**

Tissue culture medium was obtained from Life Technologies (Grand Island, NY). Reagent-grade chemicals and collagenase (type 1A) were obtained from Sigma (St. Louis, MO). Polyvinylidene difluoride (PVDF) membranes were obtained from Amersham (Arlington Heights, IL). The oxygen sensor plates, Dispase, Matrigel, and BD cell recovery solution were obtained from BD Biosciences, and renal epithelial growth medium (REGM) was obtained from Cambrex. Ouabain was purchased from Calbiochem, and bumentaniode was obtained from Biomol. Other reagents and chemicals were obtained from Sigma or Calbiochem. Anti-Tamm-Horsfall polyclonal antibodies were obtained from Biomol. Other reagents and chemicals were obtained from Sigma or Calbiochem. Anti-Tamm-Horsfall polyclonal antibodies were obtained from ICN, and anti-ROMK antibodies were obtained from ICN, and anti-ROMK antibodies were purchased from Alomone Laboratories (Jerusalem, Israel). Monoclonal antibodies against α1-Na"+-K"+-ATPase (o6f6; developed by D. Fambrough) and Na"+-K"+-2Cl"- cotransporter (T4; developed by C. Lytle) (28) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). An anti-NKCC2-specific antibody (L320), a generous gift from Dr. Mark Knepper (National Institutes of Health, Bethesda, MD) also was used for confocal microscopy and Western blot studies. A polyclonal antibody for Na"+-K"+-ATPase was obtained from Santa Cruz Biotechnology. Secondary antibodies included corresponding AlexaFluor 488-, 586-, and 594-tagged antibodies (Molecular Probes, Eugene, OR). The specificity of the antibodies was demonstrated by comparison with preimmune sera, competition after preadsorption with the appropriate peptide, or omission of primary antibodies, as reported previously (32, 40). The Biomol green reagent used in the ATPase assay was obtained from Biomol.

**Isolation of mTAL Cells**

The isolation and characterization of mTAL cells (~95% purity) were performed as previously described (6). Briefly, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital (0.65 mg/100 body wt). The kidneys were perfused with sterile 0.9% saline, via retrograde perfusion of the aorta, and cut along the corticopapillary axis. The inner stripe of the outer medulla was excised, minced with a sterile blade, and incubated for 10 min at 37°C in a 0.1% collagenase solution gassed with 95% oxygen. The suspension was sedimented on ice and mixed with Hank’s balanced salt solution (HBSS) containing 2% BSA, and the supernatant containing the crude suspension of tubules was collected. The collagenase digestion was repeated three times with the remaining undigested tissue. The combined supernatants were spun, resuspended in HBSS, and filtered through a 52-μm nylon mesh membrane (Fisher Scientific, Springfield, NJ). The filtrated solution was discarded, and the tubules retained on the mesh were resuspended in HBSS. The solution was then centrifuged at 500 rpm for 5 min, supernatant was aspirated, and the cells were resuspended in REGM.

**Propagation of a Long-Term mTAL Cell Line**

The tubule suspension was then aliquoted onto a six-well plate previously coated with a thin film of Matrigel, which was thawed out in an ice bath and diluted 1:4 with RPMI 1640 medium. With the use of chilled glassware and plasticware, the diluted Matrigel was applied to the chilled plate as a thin film, with the excess removed with a pipette. The coated plate was left in the tissue culture hood for 1 h, after which it was washed once with RPMI 1640 and was ready for use. The cells were allowed to grow to confluence, at which time they were harvested using Dispase, following the manufacturer’s recommendations, washed three times with RPMI 1640, and reseeded onto three wells of a six-well plate. Depending on the initial seeding and viability of the original cell preparation, these cultures might have required an additional subculture, after which only the medium was changed until mTAL cell colonies appeared and formed a monolayer. The time span for this latter phase was generally between 3 and 7 wk. Cells to be harvested for immunohistochemical and biochemical studies were released from the wells using BD cell recovery solution, essentially following the manufacturer’s recommendations.

**Immunocytochemistry**

For immunocytochemistry and confocal microscopy, cells were grown on polyethylene terephthalate membranes in cell culture inserts (BD Biosciences). The cells were directly seeded onto the membrane, washed several times with PBS, fixed with freshly prepared 4% paraformaldehyde in PBS for 1 h, rinsed several times with fresh PBS, and stored in the cell culture plates at 4°C. For staining, membranes were excised from the inserts and placed in 35-mm culture dishes in which all subsequent staining procedures were performed. The cells were permeabilized with 0.1% Triton X-100 in PBS for 1 h at room temperature. Primary and secondary antibodies were diluted with PBS containing 0.1% FBS. After each sequence with either a primary or secondary antibody, the membranes were washed five times with a high-salt solution containing 1% BSA and 2.3% NaCl in PBS, followed by a single wash with PBS. The membranes were placed on glass slides and mounted with Vectashield (Vector Laboratories); images were collected using an MRC 1024 ES (Bio-Rad) confocal microscopy system equipped with a black and white charge-coupled device camera and then rendered in pseudocolor. Deconvolution of collected images was carried out using the Iterative Deconvolve 3D plugin (DAMAS3 algorithm) for NIH Image J software.

**Isolation of Total RNA and RT-PCR Analysis of the NKCC2 (BSCI) Isoforms**

Total RNA was isolated from cultures of rat TAL cells or rat cortex by adding 1 ml of Trizol and incubating at room temperature for 10 min. Chloroform (0.2 ml) was then added at room temperature for 2–3 min, followed by centrifugation at 4°C at 12,000 rpm for 15 min. Isopropanol (3 vol) was added to the recovered supernatant, and the mixture was incubated at room temperature for 10 min and then centrifuged at 4°C at 12,000 rpm for 15 min. The supernatant was discarded, and the pellet was washed in 1 ml of 75% ethanol, mixed gently, and centrifuged for 5 min at 7,500 rpm at 4°C; the supernatant was removed and the pellet dried for 5–10 min. Finally, the RNA pellet was resuspended in 50 μl of RNase-free distilled H2O. After the total RNA sample was treated with deoxyribonuclease I for 30 min, a 3-μg aliquot of total RNA was used for cDNA synthesis with the SuperScript preamplification system (Life Technologies) in a 20-μl reaction mixture containing SuperScript II reverse transcriptase (200 U/μl) and random hexamers (50 ng/μl). The reaction was incubated at room temperature for 10 min to allow extension of the primers by reverse transcriptase and then at 42°C for 50 min, 70°C for 15 min, and 4°C for 5 min to obtain cDNA. The cDNA from the total RNA sample was ampliﬁed in a 25-μl PCR mixture, which contained 3 mM MgCl2, Platinum Taq polymerase (Invitrogen), and 2 μmol of specific primers.
primers (see Table 1). PCR reactions were performed in a GeneAmp System 2400 thermocycler (Applied Biosystems) in a final volume of 25 μl of dNTPs, PCR buffer, MgCl2, DNA polymerase, and specific primers. The PCR was performed in 25 μl under standard conditions [10 min of predenaturation at 95°C; 32 cycles of 40 s of denaturation at 95°C and 90 s of annealing at 55°C, respectively; 2 min of elongation at 72°C; and, finally, 7 min at 72°C] using SuperTag DNA polymerase. Negative controls included primers that were reverse transcribed in the absence of RNA, and the possibility of contamination was ruled out by including PCR control samples with no DNA template. Fragment sizes were assessed by electrophoresis in a 1% agarose gel stained with ethidium bromide. ABI Big Dye V3.0 sequencing chemistry and an ABI 3730 DNA sequencer was used to confirm the sequence of amplified fragments.

**Western Blot Analysis**

Cells were washed three times in ice-cold PBS and lysed with RIPA buffer (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). The lysates were centrifuged at 14,000 rpm for 15 min, the protein concentration of supernatants was determined using the Bio-Rad DC system, and 50 μg of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were processed with a LI-COR Biosciences system in which the membranes were blocked with LI-COR buffer for at least 1 h. The blots were incubated with primary antibodies (1:500) overnight at room temperature and with the appropriate biotinylated secondary antibody (Vector Laboratories) for 30 min. Following the manufacturer’s protocols, membranes were then developed with 1:10,000 dilution of streptavidin conjugated to infrared dye and scanned on the Odyssey infrared imaging system.

**Cl− Measurements**

6-Methoxy-N-ethylquinolinium (MEQ), a Cl−-sensitive fluorescent dye, was reduced to 6-methoxy-N-ethyl-1,2-dihydroquinoline (DiH-MEQ), which readily permeates cells (3). Inside the cells, DiH-MEQ is oxidized and converted to the cell-impermeant form (MEQ), which remains trapped in the cytosol. Polychromatic τTAL cells grown on Transwell filters were exposed to 25 μM DiH-MEQ for 20 min at 37°C and 5% CO2 in Cl−-free buffer containing 140 mM NaNO3, 5 mM KNO3, 5 mM HEPES, 1 mM Mg(NO3)2, and 5 mM glucose, pH 7.4 (9), and washed for 15 min with the same buffer to facilitate even distribution of dye within the cells. Influx of Cl− was indicated by quenching of fluorescence upon exposure of cells to a Cl−-containing buffer (same composition as the Cl−-free buffer except that NO3− was replaced with Cl−). To show that NKCC2 cotransporter mediates Cl− influx, τTAL cells were preincubated with 50 μM furosemide or vehicle control (0.001 N NaOH) for 45 min in Cl−-free buffer, followed by exposure to Cl− buffer. A FLX 800 microplate fluorescence reader (Bio-Tek) was used to measure the fluorescence with excitation at 360/40 nm and emission at 460 nm. The fluorescence intensity of MEQ undergoing collisional quenching (F) is described by the equation F = F0/(1 + KQ[Q]), where F0 is the fluorescence intensity in the absence of quencher, KQ is the quenching constant, and [Q] is the quencher concentration.

**O2 Consumption**

Confluent cells were washed three times with ice-cold PBS, and the monolayer was removed from the dish using Dispase. Cells in a 15-ml conical tube were placed on a rocking platform in the cold room for 1 h. The cell suspension was then centrifuged and washed twice with cold RPMI 1640 medium. The volume was adjusted to 1 ml with RPMI 1640 medium, and a cell count was performed. The cell density was adjusted to contain 25,000 cells in 100 μl. Cells (100 μl) were then loaded onto an oxygen biosensor plate. One hundred-microliter quantities were added to each well, containing either medium alone or medium containing a test compound. A control well was added containing medium alone. A second well contained 200 μl of 100 mM sodium sultite. The oxygen sensor plates contain a proprietary bio- sensor that fluoresces when oxygen becomes depleted. The sodium sultite serves to deplete all the oxygen and thus serves as a reference point. The plates were read on a Bio-Tek fluorescence microplate reader, set to read the bottom of the plate with excitation and emission wavelengths at 485 and 620 nm, respectively. The plate was then returned to a 37°C incubator and removed for readings at hourly intervals. The fluorescence readings were converted to O2 consumption rates according to the manufacturer’s protocol.

**Dome Formation**

Cells cultured on six-well plates formed domes as they became confluent. At this time the number of domes present was counted in several fields in each well; generally, the center and four other sections on each side of this center section. The wells were then washed several times, and the agents to be tested were added. After 4 h, the number of surviving domes was determined by recounting the slide.

**Na+−K+−ATPase Activity**

The assay is a modification of a previously described protocol based on the hydrolysis of ATP in the presence and absence of ouabain (8). Total phosphate released was determined by a microplate assay using the Biomol green reagent. The reaction mixture consists of (in mM) 37.5 imidazole, 75 NaCl, 5 KCl, 1.0 EGTA, 5 MgCl2, 6 Na3VO4, and 75 Tris·HCl (solution A) and 4 mM ATP. To assess ouabain-insensitive phosphate release, solution A was modified to contain 1 mM ouabain and 150 mM Tris, with no NaCl or KCl (solution B). Confluent cultures in six-well plates were washed twice with scraping buffer (300 mM mannitol, 10 mM Tris, 10 mM HEPES, pH 7.4) and then harvested and centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in a 1:10 dilution of streptavidin conjugated to infrared dye and scanned on the Odyssey infrared imaging system.
**Identification of raTAL Cells**

A panel of markers was used to determine whether raTAL cells were derived from the TAL tubules. Accordingly, expression of the Tamm-Horsfall glycoprotein (THP), bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC2), Na\(^+\)-K\(^+\)-ATPase, and ROMK was determined in the raTAL cell line.

THP. Expression of THP is specific for TAL cells except those that constitute the macula densa. The presence of THP was determined by indirect immunofluorescence and confocal microscopy using monoclonal and polyclonal antibodies, respectively. All cells stained positively for THP and exhibited bright staining that was observed on the plasma membrane; diffuse staining also was observed in the cytoplasm (Fig. 1). No staining was observed when cells were incubated in the absence of the primary antibody or in the presence of an isotype control primary antibody. These data are in agreement in response to ouabain and bumetanide were compared using a paired Student’s t-test.

RESULTS

**General Characteristics of the raTAL Cell Line**

Enzymatic digestion of mTAL tubules produces mTAL cells (90–95% purity) that can be maintained in culture for ~5–7 days. These tubules were used to establish a long-term polarized epithelial cell line, raTAL, by culturing the digested tubule fragments on Matrigel-coated plates and maintaining the cells in REGM at 37°C in 5% CO\(_2\). The growth conditions do not require adjustment from passage to passage, and the cells, which exhibit a homogenous appearance, are easily passaged approximately once per week after they are removed from tissue culture plates using Dispase. Cellular outgrowth from mTAL tubules derived from two kidneys leads to areas of confluent cells and dome formation within several days after seeding onto six-well plates. However, initial subculturing yielded a population of cells that were relatively quiescent for a period of 3–5 wk, after which the rate of proliferation increased. Subsequently, passages of the cultures resulted in cells that grew to confluence within 5–7 days, similar to the rate of expansion of primary mTAL cells in culture (12, 29). The cells were then maintained in culture for ~75 wk. Continual testing of the parameters described in the sections below, using cells from many different passage numbers, did not reveal any qualitative or significant quantitative differences, and the data presented are from several early and late cell passages (3-21).

**Statistical Analysis**

The responses were compared using unpaired Student’s t-test or one-way ANOVA followed by the Newman-Keuls test when multiple comparisons were made. Data are means ± SD; \( P \leq 0.05 \) was considered statistically significant. The differences in dome formation in response to ouabain and bumetanide were compared using a paired Student’s t-test.

**NKCC2**

A panel of markers was used to determine whether raTAL cells are of outer medullary origin (Fig. 2 inset). Priming for the ability of the mTAL to reabsorb ~25% of the filtered Na\(^+\) load. This transporter is only expressed in TAL cells, is active when expressed in the apical membrane, and provides an excellent marker for determining the phenotype of renal epithelial cells. Expression of NKCC2 was detected in all raTAL cells, indicating that this transporter is present for an extended period in culture (Fig. 2A). Similar results were obtained using two different antibodies: T4, which recognizes both NKCC1 and NKCC2 (shown), and an anti-NKCC2-specific antibody (L320). Moreover, Western blot analysis of raTAL cell lysates and rat renal outer medulla revealed a band of ~160 kDa (Fig. 2B).

The cell type-restricted expression of NKCC2 isoforms also provided a means of determining the phenotype of raTAL cells. Primers specific for NKCC2 isoforms A, B, and F were designed and tested in raTAL cells and rat renal cortex. RT-PCR analysis demonstrated that raTAL cells express mRNA for the A and F isoforms, consistent with the notion that these cells are of outer medullary origin (Fig. 2C, lanes 1 and 3). In contrast, mRNA for the B isoform was not detected in these cells but was detected in rat renal cortical tissue (Fig. 2C, lanes 2 and 5). Finally, raTAL cells did not express NKCC1 mRNA, which is expressed in distal segments of the nephron but not in the mTAL (Fig. 2C, lanes 4 and 6). Collectively, these data are consistent with the notion that raTAL cells are derived from the mTAL and retain the differential NKCC2 isoform expression observed in native tissue.

**Na\(^+\)-K\(^+\)-ATPase**

High levels of Na\(^+\)-K\(^+\)-ATPase are expressed in several renal cell types, including mTAL cells. This enzyme transports intracellular Na\(^+\), which has entered mTAL cells via the apical NKCC2, out of the cells through the basolateral membrane. The expression of both these proteins is a salient feature of functional mTAL cells. Indirect immunofluorescence and confocal microscopy studies revealed the typical “chicken wire” configuration for basolaterally expressed Na\(^+\)-K\(^+\)-ATPase (44) in all raTAL cells (Fig. 3). These data indicate that raTAL cells express a major transport protein that contrib-
utes importantly to Na\(^+\) reabsorption in the mTAL. Most notably, expression of the protein is restricted to the basolateral membrane, since double-labeling experiments in which cells were sequentially stained with antibodies against NKCC2 and Na\(^+\)-K\(^+\)-ATPase indicated that these proteins are not colocalized (Fig. 4).

Localization Analysis of NKCC2 and Na\(^+\)-K\(^+\)-ATPase by Confocal Microscopy

To further investigate the subcellular localization of NKCC2 and Na\(^+\)-K\(^+\)-ATPase proteins in raTAL cells, we performed a Z-series imaging experiment at 1-μm intervals beginning at the apical region of the cells and moving sequentially toward the basal region. Three-dimensional (3-D) reconstruction was accomplished using NIH Image J software and Stack Builder and Volume Viewer plugins. Two cross-sectional views from a representative Z-series analysis, one at the base of the cell and the other at the apex are shown in Fig. 5A. In the basal cross-sectional view, NKCC2 is largely intracellular and perinuclear (the nucleus is the negative image that can be seen inside the red staining for the cotransporter, filled arrowheads in Fig. 5A, left), whereas the expression of Na\(^+\)-K\(^+\)-ATPase is peripheral and lateral. However, the apical cross-sectional view shows that there was a complete red staining for the cotransporter (Fig. 5A, right, open arrowheads), suggesting that a subset of NKCC2 molecules localize to the apical region of the cell. That the green staining for the Na\(^+\)-K\(^+\)-ATPase, which has a basolateral and not apical distribution, is still exclusively on the peripheral (lateral) plasma membrane in Fig. 5A, right, serves as a control to contrast with the apical presence of the cotransporter.

The entire Z series of the images in Fig. 5A is depicted in a 3-D volume format (Fig. 5B; the apex of the cell is at the top, and the base is at the bottom). The green staining in columns indicative of Na\(^+\)-K\(^+\)-ATPase expression reveals its basolateral localization (corresponding to the peripheral localization of Na\(^+\)-K\(^+\)-ATPase in Fig. 5A). Furthermore, the NKCC2 is 1) largely intracellular and perinuclear in the basal region of the cell separable from Na\(^+\)-K\(^+\)-ATPase (corresponding to Fig. 5A, left; the filled arrowheads in Fig. 5B indicate the negative image of the nucleus inside the red staining for the cotransporter); and 2) a subset of the cotransporter actually reaches the cell apex (corresponding to Fig. 5A, right; the open arrowheads in Fig. 5B indicate apical localization of the cotransporter). Moreover, when visualized in 3-D, the red staining of the cotransporter at the apex of the cell is not intracellular and within the cell but at the same plane as the apical plasma membrane (beyond which the columns of Na\(^+\)-K\(^+\)-

Fig. 2. Expression of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter NKCC2 in raTAL cells. A: raTAL cells were grown in culture and fixed as indicated in the legend for Fig. 1 and then stained with an antibody against NKCC2 and observed by indirect immunofluorescence (original magnification, ×400) and confocal microscopy (inset). Main scale bar, 25 μm; inset scale bar, 10 μm. B: Western blot analysis of raTAL cells or rat outer medullary tissue using 2 different antibodies, L320 and T4, that recognize NKCC2. C: RT-PCR analysis of NKCC2 isoforms and NKCC1 in raTAL cells (lane 1, isoform A of NKCC2; lane 2, isoform B of NKCC2; lane 3, isoform F of NKCC2; lane 4, NKCC1) and rat cortex (lane 5, isoform B of NKCC2; lane 6, NKCC1).

Fig. 3. Expression of Na\(^+\)-K\(^+\)-ATPase in raTAL cells. raTAL cells grown and fixed as indicated in the legend for Fig. 1 were stained with a monoclonal antibody against α1-Na\(^+\)-K\(^+\)-ATPase (α6F) and examined by indirect immunofluorescence (original magnification, ×400) and confocal microscopy (inset). Main scale bar, 25 μm; inset scale bar, 10 μm.
ATPase do not rise any further). If the cotransporter were to be localized in an intracellular compartment only, then in a 3-D view there would have been a region at the apex of the cell that contained only the free green Na\(^{+}\)-K\(^{+}\)-ATPase columns, without any red staining.

**ROMK**

The apical 70 pS K\(^{+}\) channel is absent in the TAL of ROMK knockout mice, suggesting that the ROMK channel is involved in forming the apical 70 pS K\(^{+}\) channel in the TAL (27). This channel contributes importantly to K\(^{+}\) recycling at the apical membrane of the mTAL and generates a lumen-positive voltage that provides K\(^{+}\) for luminal Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport (17, 43). Abundant expression of ROMK in the cytoplasm and also at the plasma membrane was detected by both indirect immunofluorescence and confocal microscopy, indicating that an important K\(^{+}\) channel that is critical for Na\(^{+}\) reabsorption is present in raTAL cells (Fig. 6). Interestingly, unlike the other markers, ROMK was expressed in a subset of raTAL cells expressed. This finding is similar to a previous report using kidney sections, which also showed that not all mTAL cells express ROMK (46).

**Functional Analysis of raTAL Cells**

Functional analysis of the raTAL cell line was accomplished by determining Cl\(^{-}\) influx, ouabain-sensitive O\(_2\) consumption, dome formation, and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport.

**Intracellular chloride.** Influx of Cl\(^{-}\) in raTAL cells was assessed using MEQ, a quinoline derivative used as an intracellular Cl\(^{-}\) indicator based on collisional quenching of its fluorescence by halide ions (3). raTAL cells were grown on Transwell filters and loaded with DiH-MEQ in a Cl\(^{-}\)-free buffer. Cells were then preincubated with either vehicle control or furosemide (50 \text{ M}) for 45 min, and fluorescence at 360/460 nm was recorded (Fig. 7). Exposure of raTAL cells to buffer containing Cl\(^{-}\) (arrow) caused a rapid quenching of intracellular fluorescence in vehicle-treated cells that was reversible when cells were returned to a Cl\(^{-}\)-free buffer (\(\text{NO}_3\) arrow). In contrast, furosemide blocked Cl\(^{-}\) influx and prevented dye quenching (Fig. 7). The linearity of the Stern-Volmer plot (Fig. 7, insert) verifies dynamic quenching of MEQ by intracellular Cl\(^{-}\). These data indicate that raTAL cells express a functional Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, since influx of Cl\(^{-}\) in the TAL is via the bumetanide/furosemide-
sensitive cotransporter (NKCC2) expressed on the apical membrane.

**O2 consumption.** A large component of O2 consumption in mTAL cells is committed to the transport of Na+ that is directly dependent on activity of the Na+-K+-ATPase located on the basolateral membrane. Thus O2 consumption measurements were used as an index of mTAL Na+ transport, as previously validated by other investigators (7, 38). Ouabain-sensitive O2 consumption was determined using 96-well plates containing an oxygen-sensitive dye (BD Sciences). We previously showed, using a Clark-type electrode, that ouabain (1 mM), an inhibitor of the basolateral Na+-K+-ATPase, and bumetanide, a selective inhibitor of the apical Na+-K+-2Cl- cotransporter, inhibited O2 in mTAL cells by 40–60%, depending on the cell preparation (12). Preliminary experiments established that O2 consumption was directly proportional to cell number and the conditions chosen reflect a linear point on the curve (data not shown). Cells were incubated in the absence or presence of ouabain, to inhibit the Na+ pump, and O2 consumption was determined. Ouabain inhibited O2 consumption by ~42%, suggesting that a significant portion of cellular respiration is allocated for the transport of Na+ across the cell membrane (Fig. 8). Bumetanide inhibits the activity of the apical Na+-K+-2Cl- cotransporter (NKCC2), an effect that will, secondarily, lead to inhibition of the basolateral Na+ pump. Bumetanide inhibited O2 consumption by ~35% (Fig. 8). Collectively, these data are consistent with the phenotype of functionally active mTAL cells. **Dome formation.** Previous studies using time-lapse photography demonstrated that localized regions of the cell membrane lift off the bottom of the culture dish and expand to a maximum to form domes (26). Cultured mTAL cells also appear to attach to underlying plastic at localized points, and dome formation has been noted for mTAL cultures established by several investigators (10, 18, 24, 33, 34, 37, 45). The formation of these domes reflects the accumulation of salt and water molecules beneath cells grown on solid supports, which occurs following their transepithelial transport. Thus the active transport of these molecules results in localized separation of cells from the underlying matrix (26, 30, 35). Moreover, inhibition of Na+-K+-2Cl-ATPase prevents the transport of water and solutes from the apical side of the membrane through to the space between the basolateral membrane and the culture dish. An example of an area of cells expressing domes is presented in Fig. 9A. The ability of raTAL cells to transport ions in a manner consistent with polarized epithelial cells that express NKCC2 apically was determined by counting the number of domes in the absence or presence of ouabain, which was added to inhibit Na+-K+-2Cl-ATPase activity, or bumetanide, to inhibit

![Fig. 7. Measurement of Cl− influx in raTAL cells. raTAL cells were loaded with 6-methoxy-N-ethyl-1,2-dihydroquinoline and then preincubated for 45 min at 37°C in 5% CO2 with either vehicle control (0.001 N NaOH) or furosemide (50 μM). Quenching of fluorescence upon exposure to a Cl−-containing buffer was observed in vehicle but not in furosemide-treated cells. Inset represents quenching of 6-Methoxy-N-ethylquinolinium by Cl− graphed as a Stern-Volmer plot. Data are representative of 5 similar experiments.](http://ajprenal.physiology.org/)

![Fig. 8. Ouabain and bumetanide inhibit O2 consumption by raTAL cells. O2 consumption was determined in cells incubated with ouabain or bumetanide for 4 h on an oxygen biosensor plate. At the end of the incubation period, plates were read on a Bio-Tek fluorescence microplate reader set to read the bottom of the plate with excitation/emission wavelengths at 485/620 nm. *P < 0.005 (n = 4); **P < 0.0001 (n = 6).](http://ajprenal.physiology.org/)
NKCC2 activity. The number of domes was dramatically reduced after exposure of cells to ouabain for 3 h, suggesting that a ouabain-sensitive Na⁺-K⁺-ATPase contributes to the transport of salt and water from the apical to basolateral side of raTAL cells (Fig. 9B). The in vivo transport of Na⁺ via the Na⁺-K⁺-2Cl⁻ cotransporter on the apical membrane of TAL cells is linked to the activity of the Na⁺-K⁺-ATPase on the basolateral membrane. Accordingly, dome formation was evaluated after inhibition of the cotransporter with bumetanide. Addition of bumetanide inhibited the number of domes present to an extent similar to that observed when Na⁺-K⁺-ATPase activity was directly inhibited by addition of ouabain (Fig. 8C). These data suggest that the relationship between the apically localized bumetanide-sensitive cotransporter (NKCC2) and Na⁺-K⁺-ATPase is retained in the raTAL cell line.

**Na⁺-K⁺-ATPase activity.** The Na⁺-K⁺-ATPase consists of two subunits (α and β), is present in high amounts in native mTAL, and contributes importantly to the reabsorption of Na⁺, which occurs after the majority of Na⁺ enters the cell via the apical Na⁺-K⁺-2Cl⁻ cotransporter. Total cellular ATPase activity was determined by measuring the liberation of inorganic phosphate in the absence of ouabain; Na⁺-K⁺-ATPase activity was calculated as the difference between total and ouabain-insensitive ATPase activity. The specific activity of Na⁺-K⁺-ATPase was similar for primary cultures of mTAL cells and the raTAL cell line: 0.39 ± 0.08 and 0.67 ± 0.18 nmol Pi·μg protein⁻¹·min⁻¹, respectively (n = 6, P > 0.05) (Fig. 10). Na⁺-K⁺-ATPase activity in freshly isolated mTAL tubules was 1.10 ± 0.2 nmol Pi·μg protein⁻¹·min⁻¹, and the percentage of the total ATPase activity that was inhibited by ouabain was similar to that observed in other studies (2, 23, 38, 39). These data indicate that Na⁺-K⁺-ATPase activity, a critical component of mTAL function, is retained in raTAL cells.

**DISCUSSION**

We have demonstrated that a continuous line of mTAL cells (raTAL) has been derived from freshly isolated rat mTAL tubules prepared by enzymatic digestion. These cells have been maintained in culture for more than 75 passages without the loss of key functions and marker proteins that, collectively, can be used to define polarized mTAL epithelial cells. The cells retain the expression of THP, the apical bumetanide-sensitive cotransporter NKCC2, basolateral Na⁺-K⁺-ATPase, and apical ROMK. Furosemide-sensitive Cl⁻ influx, as well as ouabain- and bumetanide-sensitive O₂ consumption and Na⁺-K⁺-ATPase activity were demonstrated, indicating that transport mechanisms critical for the reabsorption of sodium are present in these cells. RT-PCR analysis demonstrated the presence of the A and F isoforms, but absence of the B isoform, of NKCC2, which is consistent with the notion that raTAL cells are derived from the mTAL.

Cell lines derived from the mTAL have been developed from rabbit and mouse kidneys. However, to the best of our knowledge, this is the first report of a rat mTAL cell line, prompted by our mTAL studies of the past two decades. Several approaches have been used to establish mTAL cell lines derived from the mTAL, and these are summarized in the figure below.
lines. Transfection of rabbit and murine TAL cells with SV40 produced cells capable of extended growth in culture (19, 33, 34). Cultures of rabbit mtAL cells also could be established without transfection if tubules were obtained from young (1 mo old) rabbits (10). The use of young (100–110 g) rats also is thought to contribute importantly to establishing the raTAL cell line. The ST-1 cell line was established by immunomagnetic purification using antiseras against THP to isolate mtAL cells from kidneys of transgenic mice expressing the SV40 large T antigen (19); this cell line expresses NKCC2 and THP, salient features of mtAL cells (25). The rabbit cell line established by Green et al. (18) and the mouse cell line by Valentich and Stoklos (37) illustrate that it is possible to maintain the mtAL phenotype after long-term culture, providing that specific conditions are established to retain the integrity of transport proteins. Similarly, proliferation of renal epithelial cells was shown to be dependent on the choice of substrate and hormonal supplementation of serum-free media (21). Microdissection of murine mtAL segments was used to develop a mouse mtAL cell line (37). These cells were maintained in culture continuously for 3 yr and were not associated with any alteration in cell morphology. Valentich and Stoklos (37) concluded that the “nature of the in vitro milieu is a significant determinant of both phenotypic stability and proliferative longevity in culture.”

raTAL cells have been sustained in culture for more than 1 yr, after careful selection of growth conditions, substrate, and a procedure for passaging the cells. The cell line was established by placing isolated tubules in bulk into six-well plates coated with Matrigel, a protocol that bears important similarities to one used to prepare mouse mtAL cell lines (37). We conclude that the choice of growth medium, substrate, and passaging protocol proved critical for establishing this cell line, facilitating the outgrowth of mtAL cells from tubule fragments at a rate that was favorable to the stability of the differentiated state. The time frame observed for initially establishing cells (3 wk) that are now being cultured on a long-term basis is similar to that observed in other studies, including a previous study from our laboratory in which a continuous culture of rabbit mtAL cells was produced (10, 21, 34). This is arguably the most critical step, since attempts to induce rapid outgrowth from tubule fragments lead to dendifferentiation of cells. Culturing mtAL cells on an appropriate substrate, as well as the contribution of critical soluble growth factors, was required to establish differentiated cell lines from rabbit mtAL (18). These conditions also proved to be critical for establishing the raTAL cell line from rat mtAL tubules. An empirical approach revealed that the combination of growing cells on Matrigel-coated plates and supporting their growth with REGM provided conditions that allowed a growth rate that enabled cells to retain structural and functional features of mtAL cells in vivo. The BD Matrigel matrix used in the present study is a solubilized basement membrane preparation extracted from a tumor rich in extracellular matrix proteins. It mimics the mammalian cellular basement membrane and contains laminin as its major component, followed by collagen IV, heparin sulfate proteoglycans, and entactin 1. At room temperature, BD Matrigel matrix polymerizes to produce biologically active matrix material resembling the basement membrane.

Positive staining for THP in all raTAL cells provides direct evidence that these cells are derived from the TAL segment of the nephron. The pattern of staining included intense staining on the plasma membrane as previously described (18, 22). Moreover, the significant diffuse staining that appears to be “cytoplasmic” is likely a function of the extensive infoldings of surface membranes associated with TAL tubules, in agreement with a previous study (18). A similar explanation is likely for the extensive intracellular staining observed for NKCC2. Although the cells were established on Matrigel-coated plates, they subsequently grow readily on membrane inserts, which have enabled visualization of apical and basolateral sorting schemes in these cells and analysis of Cl− influx in polarized cells. Accordingly, the data are consistent with localization of NKCC2 and ROMK to apical domains within the cells, in marked contrast to Na+/K+-ATPase, which is exclusively expressed in basolateral membranes. Moreover, influx of Cl− was blocked by the loop diuretic furosemide, which inhibits NKCC2. Formation of domes by raTAL cells also is significant, since it reflects the differentiated state of transporting epithelial cells. The demonstration that the number of domes observed, as well as O2 consumption, was reduced when either NKCC2 or Na+/K+-ATPase activity was inhibited supports the notion that these transporter molecules are operative in raTAL cells. Indeed, direct assessment of Na+/K+-ATPase activity indicated that this enzyme was active in raTAL cells. Collectively, these data indicate that proteins critical to mtAL function are properly oriented and functional in raTAL cells.

The kidney is a complex organ that consists of multiple cell types that function in an integrated manner. However, cellular heterogeneity can complicate attempts to ascribe specific functions to discrete cell types. Accordingly, long-term cell lines with properties similar to those of the parent cell type provide an ideal environment to study cellular and molecular function in a controlled setting. To date, most of the compendium of data available regarding the TAL and its properties has been obtained from sections of renal tissue, isolated perfused tubules, cell fractionation studies, expression of the proteins in oocytes and HEK cells, functional and immunohistochemical studies of tubules, and, in our own laboratory, from primary cultures. The present study demonstrates that a long-term epithelial cell line derived from Sprague-Dawley rats can be maintained in culture in a manner that retains essential markers and physiological functions characteristic of these cells in vivo (4, 31). These cells will be useful for studies designed to address the role of autocrine and paracrine molecules on ion transport pathways and on mechanisms that regulate the activity, expression, and trafficking of transporter molecules and will allow examination of the structural and molecular biology of determinants of mtAL function.

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Innovative Methodology

REFERENCES


