Abstract Loop-diuretic-sensitive $^{86}\text{Rb}^+(\text{K}^+)$ transmembrane fluxes were determined in cells of a mouse inner medullary collecting duct cell line (mIMCD-K2). The furosemide-sensitive (0.1 mM) influx was a substantial fraction of the total influx (0.39±0.04 or 0.42±0.03, n=5 in the presence or absence of ouabain, respectively). Furosemide also reduced $^{86}\text{Rb}^+(\text{K}^+)$ efflux by a similar fraction (0.46). RT-PCR analysis revealed expression of mRNA for the Na$^+\text{-K}^+\text{-2Cl}^–$ cotransporter-1 (NKCC1), but not NKCC2. Loop-diuretic-sensitive $^{86}\text{Rb}^+(\text{K}^+)$ influx was confined to the basolateral membrane, confirming its localisation there. The physiological properties of NKCC1 expressed in mIMCD-K2 cells, including the dependence upon medium Na$^+$, K$^+$ and Cl$^–$ and the relative sensitivity to loop diuretics as assessed by the concentration required for half-maximal inhibition (IC$\text{}_{50}$) (bumetanide 3.3±1.4×10$^{-7}$ M>piretanide 2.5±0.15×10$^{-6}$ M>furosemide 2.3±1.2×10$^{-5}$ M) were typical for NKCC1. Possible functions of NKCC1 were tested; furosemide did not inhibit the majority of forskolin-stimulated secretory short-circuit current ($I_{sc}$) (83.5±5.3% of the maintained response at 5 min). Secondly, total $^{86}\text{Rb}^+(\text{K}^+)$ influx was stimulated markedly when external osmolarity was increased to 600 mosmol/l by mannitol due to an increase via NKCC1 from 55±11 to 191±2 nmol/10$^6$ cells per 15 min, (both $n=4$, P<0.01). In contrast, 10$^{-5}$ M forskolin did not stimulate total $^{86}\text{Rb}^+(\text{K}^+)$ influx. Finally, the ability of both K$^+$ and NH$_4^+$ to compete for ouabain-insensitive $^{86}\text{Rb}^+(\text{K}^+)$ influx via NKCC1 was confirmed with similar concentrations for half-maximal influx reduction ($K_{0.5}$). Apical exposure to NH$_4^+$ elicited rapid cytosolic alkalinisation in 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-loaded epithelial layers, consistent with selective permeability of the apical membrane to NH$_3$. Conversely, NH$_4^+$ (5 mM) at the basal cell surface resulted in progressive acidification, the initial rate being reduced by 43% by furosemide. We conclude that NKCC1 participates in selective uptake of NH$_4^+$ at the basal surface, and that IMCD may function in direct NH$_4^+$ deposition to urine.

Keywords Cotransport · NKCC1 · IMCD · mIMCD-K2 cell-line · Secretion · Cell volume · Ammonium transport

Introduction

The loop-diuretic-sensitive Na$^+\text{-K}^+\text{-2Cl}^–$ cotransporter, NKCC1, is distributed widely[4, 17, 32]. In secretory epithelia the role of NKCC1 is to accumulate intracellular Cl$^–$ across the basolateral membrane above its electrochemical equilibrium level [8, 13, 17]. Upon activation of exit conductances across the apical membrane (cystic fibrosis transmembrane conductance regulator CFTR and possibly Ca$^{2+}$-activated Cl$^–$ channels), NKCC1 generates transepithelial Cl$^–$ secretion [3, 6, 8, 13]. In homozygous mutant mice in which basolateral Na$^+\text{-K}^+\text{-2Cl}^–$ cotransport has been ablated ($\text{NKCC1}^{–/–}$ animals), cAMP-induced short-circuit (secretory) currents in the intestine and in the trachea are reduced markedly [8]. More generally, NKCC1 serves to mediate ion uptake after cell shrinkage in regulatory volume increase (RVI) [17].

NKCC1 was cloned originally from a mouse inner-medullary collecting duct cell-line, mIMCD-3 [5, 17, 25], raising the question of the functional significance of NKCC1 in renal epithelia. Although it is well established that the NKCC2 isoform is responsible for transepithelial salt absorption in the thick ascending limb of the loop of Henle [17, 29], the function of NKCC1 is less well characterised. Western blotting with a polyclonal antibody against NKCC1 has shown NKCC1 to be abundant in the renal papilla and also to occur in cortex and outer medulla [16]. Immunocytochemistry using the same antibody shows localisation of the NKCC1 protein to the...
basolateral membrane of terminal IMCD segments and in the papillary surface epithelium [16]. In the outer medulla, NKCC1 is localised to α-intercalated cells [10], suggesting a role in urine acidification. Both vasopressin and hypertonicity increase NKCC1 mRNA expression in cultured mIMCD, whereas only hypertonicity increases NKCC1 protein expression [2]. Studies on both primary cultures of IMCD cells and established cell lines such as mIMCD-K2 have demonstrated that the IMCD is capable of transepithelial anion secretion, suggesting that NKCC1 has a role similar to that in other secretory epithelia [3, 5, 14, 18, 34].

Knockout NKCC1 –/– animals show a lowered blood pressure, consistent with expression of NKCC1 in vascular endothelium/smooth muscle, but do not demonstrate a profound renal deficit [8]. The purpose of this study was therefore to study more precisely the role of NKCC1 expression in the IMCD. In particular we chose to study mIMCD-K2 epithelia since this epithelium possesses both the apical cAMP-regulated CFTR and Ca2+-activated Cl– conductances [3, 20]. We determined the molecular identity of NKCC expressed in this cell-line and characterised it functionally. Surprisingly, forskolin-stimulated anion secretion dependent upon NKCC1 function and sensitive to loop-diuretic inhibition was of minor importance. This suggested an alternative role for NKCC1 in IMCD function. Accordingly, we showed that NH3 may substitute for K+ in NKCC1 function. NKCC1 contributed 43% of the total NH3 flux across the basolateral membrane, as assessed by measurement of the rate of decrease of intracellular pH (pHi) in 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-loaded mIMCD-K2 cells.

Materials and methods

Cell culture

mIMCD-K2 cells [18] were kindly provided by Dr. B. Stanton (Dartmouth Medical School, Hanover, N.H., USA). mIMCD-K2 cells were cultured routinely [3, 18, 20] in Opti-MEM with 2 mM L-glutamine and 30 µg/ml gentamycin with 10% v/v foetal bovine serum at 37 °C in an air/5% CO2 atmosphere. Stock Roux bottles (75 cm2 growth area) were coated with Vitragel/Cellon collagen and cells were passaged by trypsinisation (2 ml 0.5% w/v trypsin, 0.7 mM EDTA in Ca/Mg free saline) to form a suspension and cultured at a 1:10 split ratio. Functional epithelial layers of mIMCD-K2 cells were then cultured in six-well plates at 37 °C, 5% CO2 (2 mIMCD-K2 cells were prepared by high-density seeding (105 cells/cm2 growth area) onto collagen-coated permeable filter supports were then cultured in six-well plates at 37 °C, 5% CO2, for up to 7–25 days with medium replacement every 2–3 days. For 86Rb+ influx measurements, cells were seeded at low density (5×104 cells) onto collagen-coated Petri dishes (5 cm diameter) and cultured for 48 h.

Measurements of short-circuit current (Isc) and epithelial resistance (Ri)

Cultured epithelial layers were mounted in Ussing-type chambers maintained at 37 °C, connected to an automatic voltage clamp (DVC-1000, WPI, New Haven, Conn., USA) via KCl/agar salt-bridges and reversible electrodes (Ag/AgCl for current passage, calomel for voltage sensing) and measurements of open-circuit electrical potential difference, Ri and Isc made in modified Krebs’ solutions (see below).

Measurements of 86Rb+(K+) influx/efflux

Fluxes (influx/efflux) were measured essentially as described previously [30]. In brief, 86Rb+ was used as a tracer for K+. 86Rb+ influx (t-4 kBq/ml, 4–60 µM Rb) into sub-confluent mIMCD-K2 cells was from a modified Krebs’ solution (below) for 15 min (~8% equilibration); cell layers were then rinsed (4 times) in ice-cold Krebs’ solution to remove extracellular isotope. Intracellular isotope was then extracted into 5 ml of distilled water. 86Rb+ activity of the extracts were obtained by Cerenkov counting in a liquid scintillation spectrometer. Parallel mIMCD-K2 cell culture plates were treated with a trypsin solution (0.25% w/v) in a Ca2+-Mg2+-free medium to form a cell suspension for determination of cell number/plate. Fluxes were expressed in nanomoles/106 cells per 15 min. For certain experiments influx was determined into confluent epithelial layers from either the apical or basal compartment alone. Transepithelial flux of 86Rb+ into the contralateral compartment was 1% of the total present in the apical or basal compartment. For 86Rb+ efflux, subconfluent cell layers were preloaded with 86Rb+ for 2–3 h, rinsed 4 times in Krebs’ solution to remove extracellular isotope, and efflux determined by sequential replacement of 2 ml of Krebs’ at 37 °C every 5 min for up to 1 h. Remaining intracellular isotope was then extracted into 5 ml distilled water. Efflux for every 5-min period is then expressed as a fraction of the total intracellular isotope for that period [30].

Measurements of pHi

mIMCD-K2 cells grown as epithelial monolayers were loaded by incubation with 10 µM BCECF-acetoxymethylester (BCECF-AM) in the apical bathing solution in standard growth media for 15–30 min at 37 °C in a 5% CO2/95% air atmosphere. The epithelial layers were then placed in a 24-mm diameter perfusion chamber fitted to the stage of an inverted Nikon Diaphot fluorescence microscope. The chamber allowed perfusion of both apical and basal sides. The cells were imaged using a long-working-distance objective (Nikon ×40), and perfused continuously (bath volume apical 0.5 ml, basal solution 1 ml perfusion at 5 ml/min).

Changes in pHi were determined by measuring the fluorescence of the BCECF-loaded cells with a dual-wavelength excitation micro-spectrofluorimeter (Newcastle Photometrics, UK). Groups of cells (between five and ten) were illuminated alternate-ly with excitation light at 440 and 490 nm (cycle time 1.2 s) and emitted light was filtered using a 520 nm long-pass filter. Fluorescence of the BCECF-loaded cells was detected using a dual-wavelength excitation micro-spectrofluorimeter (Newcastle Photometrics, UK). The fluorescence emission ratio with pH was made using cell-layers pretreated with 10 µM nigericin and superfusing both the apical and basal solutions with a high KCl (150 mM) buffer, the pH of which was set between 6.0 and 9.0 using combinations of 3-(N-morpholino)propanesulfonic acid (MOPS), 2-(N-morpholino)ethanesulfonic acid (MES), HEPES and TRIS [33]. Results are expressed as ΔpHi. Calculations of the effect of drug effects on NH4+-induced change in pHi were made by linear regression using NPS system v. 4.7 software.

Expression and identity of mNKCC1 mRNA-related transcripts by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was obtained from whole mouse kidney using the acid phenol extraction reagent RNAzol B (Biogenesis) as follows: tissue was ground in liquid N2 and added to 20 ml RNAzol B. The suspension was homogenised using a Kinematica CH-6010 homogeniser. Chloroform was added (2 ml) and the sample shaken vig-