K-Cl COTRANSPORT: ROLE OF KCC3 IN CELLULAR POTASSIUM (K) HOMEOSTASIS IN KCC3-TRANSFECTED HEK-293 CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

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Ravilla, Nagendra Babu. M.S. Department of Pharmacology and Toxicology, Wright State University, 2013. K-Cl cotransport: Role of KCC3 in cellular potassium (K) homeostasis in KCC3-transfected HEK-293 cells.

K-Cl cotransport (KCC) mediated by four protein isoforms, KCC1 to KCC4, plays a significant role in cell volume regulation, and in K and Cl homeostasis. In this study, we demonstrate the importance of KCC3 and its two threonine (T) phosphorylation sites T991 and T1048 in cellular K homeostasis in isogenic human embryonic kidney (HEK-293) cells transfected with KCC3 wild type (WT) or constitutively active double mutant KCC3 (AA) expressed under tetracycline or doxycycline control. In both WT and AA cells, under baseline conditions, the Na-K-2Cl cotransport (NKCC) (55 %- 60 %) was the major contributor of Rb influx followed by the Na/K pump (NKP) (35 % - 40 %) and KCC (10 % - 15 %) even though the absolute values of Rb influx were higher in AA cells. Doxycycline induction had no effect on either NKCC or KCC in WT cells, whereas NKCC was completely inhibited and KCC was increased by 25 fold in AA cells. In AA cells, intracellular K (Kᵢ) decreased by 90 % upon doxycycline induction in Cl medium and 50 % of this loss was attenuated in Cl-free (sulfamate replacement) medium. Doxycycline-induced Cl-dependent Kᵢ loss was sensitive to furosemide (2 mM) but insensitive to tetra ethyl ammonium (TEA) (2 mM) and to changes in external K and Na, whereas doxycycline-induced Cl-independent Kᵢ loss was sensitive to 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB) (50 µM) but insensitive to TEA (2 mM), furosemide (2 mM) and extracellular K and Na.
Table of Contents

1. Introduction ......................................................................................................................... 1
   1.1. K-Cl cotransport (KCC) ................................................................................................. 2
   1.2. Significance of KCC ....................................................................................................... 3
   1.3. Mechanisms regulating KCC .......................................................................................... 6
   1.4. KCC3 structure .............................................................................................................. 9
2. Hypothesis and Specific Aims ............................................................................................. 11
   2.1. Background for hypothesis ............................................................................................ 11
   2.2. Hypothesis .................................................................................................................. 11
       2.2.1. Specific aim 1 ........................................................................................................ 12
       2.2.2. Specific aim 2 ........................................................................................................ 12
       2.2.3. Specific aim 3 ........................................................................................................ 12
3. Materials and Methods ........................................................................................................ 13
   3.1. Chemicals ...................................................................................................................... 13
   3.2. Inhibitors ..................................................................................................................... 13
   3.3. Solutions for flux studies .............................................................................................. 14
   3.4. KCC3 transfections ....................................................................................................... 15
   3.5. Cell cultures ................................................................................................................ 15
   3.6. Ion flux studies .............................................................................................................. 15
4. Results ........................................................................................................................................... 17

4.1. Baseline Rb (K) influx components in KCC3 WT and AA cells .................. 17

4.2. Doxycycline-induced Rb (K) influx in WT and AA cells ....................... 18

4.3. Doxycycline-induced Rb (K) influx as a function of time ...................... 18

4.4. Physiological factors affecting Rb influx in WT and AA cells.............. 21

4.5. Intracellular K (K_i) in WT and AA cells ............................................. 23

4.6. Effect of extracellular K (K_o) on K_i loss in Na medium .................. 27

4.7. Effect of K_o on K_i loss in Na-free medium ...................................... 28

4.8. Effect of furosemide on Cl-dependent K_i loss .................................. 29

4.9. Effect of TEA on K_i loss ................................................................. 30

4.10. Effect of DCPIB on K_i loss ........................................................... 31

5. Discussion ................................................................................................................................. 33

6. Acknowledgements .................................................................................................................. 36

7. References ............................................................................................................................... 37
<table>
<thead>
<tr>
<th>Figure Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Model for cell volume regulation</td>
<td>7</td>
</tr>
<tr>
<td>Figure 2. Proposed KCC regulation mechanism by vasodilators in vascular smooth</td>
<td></td>
</tr>
<tr>
<td>muscle cells(VSCMs)</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3. Proposed mechanism of KCC regulation by platelet-derived growth factor</td>
<td>9</td>
</tr>
<tr>
<td>(PDGF) in VSMCs</td>
<td></td>
</tr>
<tr>
<td>Figure 4. Hypothetical model of KCC3 structure</td>
<td>10</td>
</tr>
<tr>
<td>Figure 5. Baseline Rb (K) influx components and transporters for WT and AA cells</td>
<td>17</td>
</tr>
<tr>
<td>Figure 6. NKCC and KCC in WT and AA cells before and after doxycycline induction.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 7. Rb uptake as function of time in WT and AA cells</td>
<td>20</td>
</tr>
<tr>
<td>Figure 8. Rb influx in Na and Na-free (NMDG) medium in WT and AA cells</td>
<td>22</td>
</tr>
<tr>
<td>Figure 9. Effect of serum deprivation on NKCC and KCC in WT and AA cells</td>
<td>22</td>
</tr>
<tr>
<td>Figure 10. Effect of tonicity on NKCC and KCC in WT and AA cells</td>
<td>23</td>
</tr>
<tr>
<td>Figure 11. Intracellular K content ($K_i$) in WT and AA cells for control and doxycycline-induced cells</td>
<td>24</td>
</tr>
<tr>
<td>Figure 12. Time-course of $K_i$ content in WT and AA cells</td>
<td>24</td>
</tr>
<tr>
<td>Figure 13. Hypothetical model for doxycycline-induced K loss in KCC3 (AA) mutant</td>
<td>26</td>
</tr>
<tr>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>Figure 14. Approaches to inhibit $K_i$ loss</td>
<td>27</td>
</tr>
<tr>
<td>Figure 15. Effect of extracellular K($K_o$) on $K_i$ in Na medium</td>
<td>28</td>
</tr>
</tbody>
</table>
Figure 16. Effect of $K_o$ on $K_i$ in Na-free medium ................................................................. 29

Figure 17. Effect of 2 mM furosemide on $K_i$ loss ($K$) and Rb influx (Rb) ......................... 30

Figure 18. Effect of TEA on $K_i$ loss ($K$) and Rb influx (Rb) ................................................. 31

Figure 19. Effect of DCPIB on $K_i$ loss ($K$) and Rb influx (Rb) ............................................. 32
List of Tables

Table 1. K-Cl cotransport: Gene expression and significance ........................................5
1. **Introduction**

Regulation of cell volume is vital for cell survival. Lacking a rigid cell wall, animal cells are more vulnerable to changes in extracellular or intracellular osmolality; except in a few cell types, the cell membrane is highly permeable to water. However, compared to the water permeability, the cell membrane’s permeability to Na$^+$, K$^+$ and Cl$^-$ is orders of magnitude lower. Thus to maintain a stable cell volume, both prokaryotic and eukaryotic cells have evolved with numerous transporter proteins that transport Na$^+$, K$^+$ and Cl$^-$ across the plasma membrane. Regulation of these transporters is a highly complicated physiological phenomenon involving several physical, chemical and biological factors that act harmoniously with the ultimate aim to preserve membrane integrity and cell volume.

According to the “pump and leak” hypothesis [8], cell swelling and lysis are avoided by a limited permeability of the plasma membrane to Na, and by a continuous Na-K-ATPase activity, which pumps Na out and K in. Most cells effectively counteract changes in cell volume following perturbation in intracellular or extracellular osmolality either by regulatory volume increase (RVI) or regulatory volume decrease (RVD) in which several transporters and channels are involved [14, 48].

In osmotically swollen cells, K, Cl and other nonessential organic osmolytes exit the cell followed by water, and this leads to the recovery of cell volume. This mechanism is called RVD, which involves volume-sensitive Cl and K channels along with electro-neutral KCC. In contrast, when a cell shrinks due to osmotic shock, Cl, K and Na enter
the cell followed by water and thus restore the cell volume. This mechanism is defined as RVI, which involves the Na/H exchanger, NKCC, and non-selective cation channels [14].

In this study, we explore the role of KCC in K and Cl homeostasis and cell volume regulation in a human epithelial cell line by using KCC3 wild type (WT) and KCC3 mutant (AA) transfected human embryonic kidney (HEK-293) cells. Before providing a more detailed description of the study, a brief introduction about K-Cl cotransport and its regulation and significance will be presented.

1.1. K-Cl cotransport (KCC)

KCC is a secondary active transport that extrudes K and Cl out of the cell, preserving intracellular Cl and K homeostasis and thus cell volume. This transport system was first identified as an N-ethylmaleimide (NEM) - and swelling activated K efflux in low K sheep red blood cells [42, 44]. It is encoded by four genes SLC12A4 to 7 and thus has four protein isoforms KCC1 to KCC4 with splice variants in each isoform. KCC1, being present in all the tissues, is considered as housekeeping KCC isoform [5, 38], whereas KCC2 is mainly restricted to the central nervous system [18, 38], even though recent studies confirmed its presence in lens epithelial cells [43], retinal neurons [20, 38] and cervical cancer cells [59]. More recently, a second spliced variant of KCC2 has been reported [52]. Therefore, the originally published KCC2 of the then neuronal system is now the KCC2b variant, whereas the second and longer variant KCC2a occurs not only in the brain but also in other tissues [47, 52]. KCC3 presence was reported for brain, heart,
skeletal muscle and kidney [7, 25, 38]. KCC3 has NH$_2$– terminal heterogeneity with two major isoforms, a longer KCC3a, and shorter KCC3b, which are highly expressed in kidney (1). The KCC4 protein was discovered in several tissues like bone marrow, spleen, thymus, spinal cord, brain, prostate, pancreas, liver, skeletal muscle, heart lung and the basolateral membranes of the nephron [7, 38]. The main function of these transporters is regulation of cell volume by RVD [48]. When the extracellular milieu becomes hypoosmotic, water enters the cell resulting in increased cell volume. This increased cell volume activates the volume-sensitive K and Cl channels along with KCCs and extrudes the K and Cl ions followed by water to bring the cell back to its original volume [48].

1.2. **Significance of KCC**

Besides regulating the cellular volume, KCC has been implicated in several pathologies and in cell growth and migration. Several reviews give us a clear idea about the importance of KCC in sickle cell disease, cardiovascular pathology, epileptic seizures and tumor biology [39, 10, 11, 21, 57]. Numerous studies have shown the importance of KCC1 in cellular dehydration of red blood cells (RBCs) in homozygous sickle cell disease [45, ref. in].

KCC2 being neuron specific has been reported to play an important role in deciding the neurotransmitters GABA$_A$ and glycine function. During early fetal life, GABA$_A$ and glycine act as excitatory neurotransmitters due to high intracellular Cl (Cl$_i$) whereas in adult neuron, expression of KCC2 results in decreased Cl$_i$ and this leads to inhibitory action of GABA$_A$ and glycine [3, 6, 10]. KCC2 knockout mice cannot live and die of respiratory failure [19]. Heterozygous mice with reduced KCC2 expression are susceptible to hyper-excitability and epileptic seizures demonstrating the role of KCC2 in epilepsy [41].
Mutations in KCC3 are involved in an autosomal recessive syndrome called Anderman syndrome in which agenesis (A) of carpus (C) callosum (C) is accompanied by a peripheral (P) neuropathy (N) (ACCPN) [17], which is mostly seen in the French-Canadian population of the Quebec region of Canada. People with this disorder suffer from areflexia, hypotonia, amyotrophy and tremors due to improper development of certain cranial nerves. KCC3 knockout mice exhibit arterial hypertension [30, 40]. KCC3 knock out in mice also results in progressive neurodegeneration, deafness and decreased threshold for seizure or epilepsy [55]. A study in KCC3-transfected NIH/3T3 fibroblasts shows the role of this transporter in cell growth and proliferation [34]. Recent studies also exploit the role of KCC, and specifically KCC3 and KCC4, in cancer cell invasion and proliferation [58, 560, 59]. Besides its role in tumor biology, KCC4 is also important in acid-base balance because mutations in KCC4 result in renal tubular acidosis [54].
### Table 1. K-Cl cotransport: Gene expression and significance (Modified from ref.21)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SLC12A4</th>
<th>SLC12A5</th>
<th>SLC12A6</th>
<th>SLC12A7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Gene Locus</td>
<td>16q22</td>
<td>20q13</td>
<td>15q14</td>
<td>5p15</td>
</tr>
<tr>
<td>Protein</td>
<td>KCC1</td>
<td>KCC2</td>
<td>KCC3</td>
<td>KCC4</td>
</tr>
<tr>
<td>Isoforms and Amino Acid Residues</td>
<td>1085</td>
<td>KCC2a-1075 KCC2b-1115</td>
<td>KCC3a-1150 KCC3b-1099</td>
<td>1083</td>
</tr>
<tr>
<td>Tissue Expression</td>
<td>Ubiquitous</td>
<td>Neurons and other tissues</td>
<td>Extensive</td>
<td>Extensive; Limited in Brain</td>
</tr>
<tr>
<td>Direct Disease Association</td>
<td>-</td>
<td>-</td>
<td>ACCPN (Anderman Syndrome)</td>
<td>-</td>
</tr>
<tr>
<td>KO Phenotype</td>
<td>No phenotype</td>
<td>Death by respiratory failure, incomplete knock-out-epilepsy, death in 15 days</td>
<td>Hypertension, deafness, tumor biology</td>
<td>Renal tubular acidosis, epilepsy, sickle cell anemia</td>
</tr>
<tr>
<td>Importance in Complex Disease</td>
<td>Sickle cell anemia?</td>
<td>Epilepsy</td>
<td>Renal tubular acidosis, epilepsy, sickle cell anemia</td>
<td>Renal tubular acidosis, epilepsy, sickle cell anemia</td>
</tr>
</tbody>
</table>
1.3. Mechanisms regulating KCC

The role of numerous signaling pathways in regulating KCC activity has been elucidated. Phosphorylation/dephosphorylation of the transporter through series of kinases/phosphatases is the major mechanism that regulates this transporter as initially proposed by Jennings [33]. Phosphorylation inhibits KCC and activates NKCC and vice versa via dephosphorylation. Studies during the last decade have elucidated the essential role of “with no lysine kinases” (WNKs) in the regulation of NKCC and KCC (26, 27, 28, 29). NKCC regulation by WNK kinases via Ste20/Sps1-related proline/alanine rich kinase (SPAK) and the oxidative stress responsive kinase 1(OSR1) has been well explored compared to KCC regulation through these kinases (24, 11). Regulation of NKCC and KCC via these kinases and their role in cell volume regulation is shown in figure 1. Other major mechanisms include the regulation by the NO/cGMP/PKG pathway [31, 38] and PDGF-mediated pathway in vascular smooth muscle cells (VSMCs) [32, 36, 38 and ref. in] and the phosphoinositol/PKC pathway in low-K sheep red cells which have KCC1 and KCC3 isoforms [4, 38].
Figure 1. Model for cell volume regulation. Role of osmosensitive kinases and phosphatases and of NKCC, and KCC in cell volume regulation via RVI and RVD. Details are discussed in text.
Figure 2. Proposed KCC regulation mechanism by vasodilators in vascular smooth muscle cells. Vasodilators (VDs) such as sodium nitroprusside (SNP) release nitric oxide (NO) and activate the soluble guanylyl cyclase (sGC). The enzyme converts GTP into cGMP and this, in turn, activates protein kinase G (PKG) which, by an unknown mechanism, activates protein phosphatase 1 (PP1) and KCC. SNP and 8-Br-cGMP, NO donor and PKG activator, respectively, stimulate the KCC, whereas sGC and PKG antagonists LY 83583 and KT 5823, respectively, inhibit KCC. (Redrawn from [36])
Figure 3. Proposed mechanism of KCC regulation by platelet-derived growth factor (PDGF) in VSMCs. PDGF binds to its receptor and activates PI 3-K which, through an unknown mechanism, activates PP1, leading to dephosphorylation and activation of K-Cl COT. Involvement of the PDGF receptor was uncovered by inhibition with AG1296. Participation of PI 3-K was determined by inhibition with Wortmannin and LY294002. Finally, participation of PP1 was studied with calyculin A, a selective inhibitor of the phosphatase. (Redrawn from [36]).

1.4. KCC3 structure

As represented in figure 2, human KCC3 is a trans-membrane protein with KCC3a and KCC3b isoforms constituting 1150 and 1099 amino acid residues, respectively, with molecular weights of the un-glycosylated proteins of 130 and 125 kDa, respectively, possessing an intracellular short N-terminal and long C-terminal, and an extracellular loop connecting trans-membrane domains 5 and 6. KCC3 bears several glycosylation sites on its extracellular loop whose functions are still unknown. Recent
studies revealed that KCC3 has two functionally significant phosphorylation sites; threonine (T) 991 and 1048; their deletion or mutation perturb KCC activity [22].

Figure 4. Hypothetical model of KCC3 structure. KCC3 is reported to contain 12-transmembrane domains, a larger extracellular loop between domains 5 and 6, and intracellular N- and C-terminal domains, in addition to phosphorylation and glycosylation sites. Details are discussed in the text.
2. **Hypothesis and Specific Aims**

2.1. **Background for hypothesis**

The two functionally important threonine (T) kinase phosphorylation sites in KCC3, T991 and T1048, are localized on its C-terminal. The double A substitution prevents phosphorylation, which inhibits KCC3 at these sites, and hence activates the transporter. Thus, the AA mutant mimics the dephosphorylated KCC3 that is present during RVD [22]. We detected permanent activation of KCC3 through increased Rb (K) influx. Preliminary data from our experiments showed that increased Rb (K) influx was associated with K loss in KCC3AA-transfected HEK-293 (AA) cells. Based on these data, we propose the following hypothesis.

2.2. **Hypothesis**

The threonine (T) phosphorylation sites T991 and T1048 on KCC3 are important for K-Cl cotransport regulation. Their replacement with alanine (A) permanently activates KCC3 as measured by Cl-dependent Rb influx and K-loss. Expression in HEK-293 cells of the AA-mutant KCC3 also may result in collateral stimulation of K loss mechanisms and perturb cellular K homeostasis.
2.2.1. **Specific Aim 1**

Characterize baseline and doxycycline-induced Rb (K) influx under physiological conditions in the presence and absence of Cl anions, and thus K-Cl cotransport, in WT and AA transfected HEK293 cells to determine whether AA cells are indeed permanently activated.

2.2.2. **Specific Aim 2**

Determine whether AA cells are indeed permanently activated by assessing the role of confounding factors such as external Na, serum deprivation and osmolality on Rb influx.

2.2.3. **Specific Aim 3**

Identify K loss mechanisms induced by doxycycline in AA cells utilizing ion substitution and a variety of chemical inhibitors of K, Cl and Ca channels.
3. Materials and Methods

3.1. Chemicals

Chemicals from Fisher Scientific (Fair lawn, NJ) were: Tri-hydroxyamino methane (Tris) free base, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), sodium hydroxide (NaOH), sucrose, D-glucose, perchloric Acid, 70 % (PCA), and bicinehonic acid (BCA) protein assay reagents. Magnesium gluconate was from Sigma-Aldrich (St. Louis, MO). (4-((2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) free acid, and calcium chloride (CaCl₂), anhydrous were from J.T.Baker Chemical Co (Center valley, PA). Rubidium chloride (RbCl), 99.8 % (metals basis), and amidosulfonic acid (sulfamic acid, S), 99.99 % (metals basis) were purchased from Alfa Aesar (Ward Hill, MA); N-methyl D-glucamine (NMDG) from Fluka Biochemika (St. Louis, MO); Cesium chloride (CsCl) from Life technologies (Carlsbad, CA) and calcium gluconate from Acros Organics (NJ).

3.2. Inhibitors

Ouabain octahydrate was purchased from Calbiochem (San Diego, CA), furosemide and bumetanide from Sigma-Aldrich (St. Louis, MO), DCP1B from Tocris Bioscience (Bristol, UK), tetra ethyl ammonium (TEA) from Abcam (Cambridge, MA), clofilium tosylate from Enzo life sciences (Farmingdale, NY), and RN-1734 and Ruthenium Red from Santa Cruz Biotechnology (Santa Cruz, CA).
3.3. Solutions for flux studies

Washing solutions

Balanced salt solution (BSS-NaCl): 20 mM Hepes-Tris, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and NaCl, pH 7.4, 37 °C, 300 mOsM.

BSS-NaS: 20 mM Hepes-Tris, 5 mM K sulfamate, 2 mM Ca gluconate, 1 mM Mg gluconate, 10 mM glucose and NaS. pH 7.4, 37 °C, 300 mOsM.

Pre-incubation solutions

BSS-NaCl-BSA: 20 mM Hepes-Tris, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.1 % BSA and NaCl, pH 7.4, 37 °C, 300 mOsM.

BSS-NaS-BSA: 20 mM Hepes-Tris, 5 mM K sulfamate, 2 mM Ca gluconate, 1 mM Mg gluconate, 10 mM glucose, 0.1 % BSA and NaS. pH 7.4, 37 °C, 300 mOsM.

Flux solutions

BSS-RbCl-NaCl-BSA: 20 mM Hepes-Tris, 10 mM RbCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.1 % BSA and NaCl, pH 7.4, 37 °C, 300 mOsM.

BSS-RbS-NaS-BSA: 20 mM Hepes-Tris, 10 mM Rb sulfamate, 2 mM Ca gluconate, 1 mM Mg gluconate, 10 mM glucose, 0.1 % BSA and NaS. pH 7.4, 37 °C, 300 mOsM.

Final wash solution

10 mM MOPS-Tris, MgCl₂, pH 7.4, 37 °C, 300 mOsM.

Rb Extraction

5 % perchloric acid, 4 mM CsCl

Protein dissolution

1M NaOH
3.4. **KCC3 transfections**

Single copies of WT or AA human KCC3a cDNAs with an N-terminal myc epitope stably expressed under control of tetracycline were transfected into HEK-293 cells using Flip-in T-Rex System (Invitrogen). In this study, doxycycline, a tetracycline derivative, was used to induce gene expression.

3.5. **Cell cultures**

Human embryonic kidney (HEK293) cells stably transfected with WT or AA KCC3a cDNAs were cultured in Dulbecco's Modified Eagle (DMEM) high glucose medium (Life Technologies, Catalog No.11965-092) supplemented with 10% tetracycline-free fetal bovine serum (FBS) (Clontech, Catalog No.631106), 100 units/ml penicillin (Hyclone, Catalog No. SV30010), 100 μg/ml streptomycin (Hyclone, Catalog No. SV30010) and 10 μg/ml blasticidin S hydrochloride (Santa Cruz, Catalog No. sc-204655) in a humidified incubator with 5% CO₂ at 37°C.

3.6. **Ion Flux Studies**

Ion fluxes were determined according to previously published protocols (23, 35, 37) with some modifications. Cells were grown to 60% confluence in 12-well plates; KCC3 was expressed by overnight doxycycline (1μg/ml) induction. WT- or AA-treated cells were removed from the incubator, culture medium was aspirated, washed three times with 1ml 300 mOsM BSS-NaCl or BSS-NaS and then equilibrated in BSS-BSA-NaCl or BSS-BSA-NaS, pH 7.4, 37°C for 10 min, thereafter supernatants were removed and then exposed to pre-warmed actual flux media BSS-BSA-RbCl or BSS-BSA-RbS for pre-determined time points (2.5 min to 15 min). Thereafter, cells were washed with washing
solution at room temperature to block Rb influx or K\textsubscript{i} loss, cellular Rb and K were extracted after cell lysis by exposing for 15 min to ice cold 5 \% PCA and finally protein was extracted by dissolving in 1 M NaOH. Rb and K were measured by atomic emission and absorption spectrophotometry, respectively using a Perkin Elmer 5000 atomic absorption spectrophotometer. Protein was determined by the BCA assay. Rb uptake or K\textsubscript{i} loss was calculated in nmoles/mg protein as function of time. Ouabain (0.1 mM) and bumetanide (10 \(\mu\)M) were added to the flux media to block NKP and NKCC, respectively. Ouabain- and bumetanide-sensitive Rb influx in Cl\textsuperscript{−} was calculated as NKP and NKCC, respectively, whereas Cl\textsuperscript{−}-dependent Rb influx in presence of ouabain and bumetanide was determined as KCC.
4. Results

4.1. Baseline Rb (K) influx components in KCC3 WT and AA cells

Figure 5. Baseline Rb (K) influx components and transporters for WT and AA cells. Flux time was 5 min and Rb influx components and transporters were calculated as described in Materials and Methods. n = 9 for WT cells, and n = 6 for AA cells; values are means ± S.E. Details are discussed in the text.
Baseline NKP, NKCC and KCC were calculated by performing unidirectional uptake experiments using Rb as K congener in KCC3 WT- and AA-transfected HEK-293 cells. The transporter activities were calculated as the Rb influx in (nmoles/mg protein) as shown in [Fig.5]. In WT cells, NKP and NKCC contributed to 90 % of the total Rb influx whereas only 10 % of influx occurred through KCC. The major contributor of the Rb influx was NKCC (50 %) followed by NKP (40 %). In AA cells, baseline NKCC activity was 58-60 % of the total Rb influx followed by NKP (28-30 %) and KCC (10-12 %), which are basically similar to the values observed in WT cells. Furthermore, under baseline conditions NKCC was the major component of Rb (K) influx in both WT and AA cells and was slightly more active in AA cells, whereas KCC activity was quite similar in both types of cells.

4.2. Doxycycline-induced Rb (K) influx in WT and AA cells

Rb influx was measured after overnight gene expression under doxycycline control. NKCC and KCC were calculated and results are shown in [Fig.6]. Doxycycline did not induce significant changes on NKCC and KCC activities in WT cells, whereas in AA cells, it induced a 25 fold increase in KCC activity. RbCl uptake was larger in the presence of ouabain and bumetanide compared to the uptake in the presence of ouabain alone and thus it resulted in calculated negative NKCC values, which have no physical meaning.

4.3. Doxycycline-induced Rb (K) influx as function of time

The doxycycline-induced RbCl uptake was calculated as a function of time in WT and AA cells and the results for 5 min flux are shown in [Fig.5]. Furthermore, in WT cells, NKCC and NKP activities were non-linear even at the earliest time point tested (i.e. 2.5 min) whereas KCC activity was linear up to 15 min [Fig.7]. In AA cells, KCC activity
was non-linear and saturated at 10 min, whereas NKCC activity became more negative with increasing time (negative values have no physical meaning as discussed earlier) that could be due to an increase in Rb uptake in the presence of bumetanide.

Figure 6. NKCC and KCC in WT and AA cells before and after doxycycline induction. NKCC and KCC were calculated as the Cl-dependent ouabain-insensitive, bumetanide-sensitive and Cl-dependent (ouabain + bumetanide)-insensitive Rb influx (nmoles/mg protein x 5 min), respectively. n = 9 for WT cells and n = 4 for AA cells. Values are means ± S.E. Details are discussed in the text.
Figure 7. Rb uptake as function of time in WT and AA cells. Rb transporters NKP, NKCC and KCC were calculated as described in Materials and Methods. n = 9; values are means ± S.E. Details are discussed in the text.
4.4. Physiological factors affecting Rb influx in WT and AA cells

To better understand the response of Rb (K) influx both in WT and AA mutants; we tested the effect of physiological factors such as external Na, serum and osmolality in these cells, [See Figs. 8, 9 and 10, respectively]. To assess the effect of external Na ($Na_o$) on Rb influx, N-methyl D-glucamine (NMDG) was used as Na replacement. As expected, in both WT and AA cells the Na-dependent transporter NKCC was completely inhibited in Na-free medium, whereas KCC was not affected in WT cells. Interestingly, in AA cells, the doxycycline-induced KCC activity was reduced by 30 % in the absence of external Na. Furthermore, serum deprivation for 24 h had no statistically significant effect on KCC activity under baseline and doxycycline-induced conditions both in WT and AA cells, in contrast to previous observations (36 and ref. therein), whereas NKCC was inhibited by 25 % under baseline conditions and, as shown earlier, it was abolished in doxycycline-induced cells. In general, hypertonicity activates NKCC and inhibits KCC and hypotonicity inhibits NKCC and activates KCC. In WT cells, NKCC was activated by both hypertonicity and hypotonicity and KCC was unaffected (Fig. 10WT). This result could be outcome of variable ionic strength in flux media. In AA cells neither NKCC nor KCC was affected by changes in osmolality (Fig.10AA). This result was expected because permanently activated KCC3 is insensitive to changes in osmolality [22].
Figure 8. Rb influx in Na and Na-free (NMDG) medium in WT and AA cells. NKCC and KCC represent Rb influx in the absence of doxycycline, and DNKCC and DKCC represent Rb influx after overnight doxycycline induction. n = 6; values are ± S.E. Details are discussed in the text.

Figure 9. Effect of serum deprivation on NKCC and KCC in WT and AA cells. NKCC and KCC are controls, DNKCC and DKCC in the presence of doxycycline. Flux time, 5 min; n = 4 for WT cells and n = 6 for AA cells. Values are means ± S.E. Details are discussed in the text.
Figure 10. Effect of tonicity on NKCC and KCC in WT and AA cells. Doxycycline induced NKCC and KCC activity in hypotonic (200 mOsM), isotonic (300 mOsM) and hypertonic (450 mOsM) flux media with variable ionic strength. Flux time 5 min. n = 6; values are means ± S.E. Details are discussed in the text.

4.5. Intracellular K ($K_i$) in WT and AA cells

Figure 11. shows $K_i$ in WT cells, when 15 min measured after exposure to Cl or S media in the presence of ouabain and of [ouabain + bumetanide]. Note that in S, there was a 30% $K_i$ loss with respect to ClO or ClOB and was not affected by doxycycline treatment. By definition, $K_i$ loss in sulfamate indicates activation of a Cl-independent $K_i$ loss mechanism. Surprisingly, induction of KCC3 by doxycycline in AA cells caused a 75% $K_i$ loss in Cl, 50% of which was recovered by placing the cells in S medium, as shown in (Fig. 11 AA), indicating activation of a Cl-dependent $K_i$ loss mechanisms through the KCC. Furthermore, increase in flux time from 2.5 min to 10 min increased the doxycycline-induced Cl-dependent $K_i$ loss but no significant change was observed in the Cl-independent $K_i$ loss (Fig. 12 AA).
Figure 11. Intracellular K content (K_i) in WT and AA cells for control and doxycycline-induced cells. K_i after flux in 10 mM RbCl with 0.1 mM ouabain (ClO); with 0.1 mM ouabain + 10 µM bumetanide (ClOB); – and 10 mM RbS with 0.1 mM ouabain + 10 µM bumetanide (SOB). Flux time, 5 min; n = 9; values are means ± S.E. Details are discussed in the text.

Figure 12. Time-course of K_i content in WT and AA cells in the absence and presence of doxycycline (D). K_i after flux in 10 mM RbCl, with 0.1 mM ouabain (ClO); with 0.1 mM ouabain + 10 µM bumetanide (ClOB); – and 10 mM RbS with 0.1 mM ouabain + 10 µM bumetanide (SOB). Flux time, 5 min; n = 9; values are means ± S.E. Details are discussed in the text.
This doxycycline-induced unidentified and significantly large $K_i$ loss in isotonic conditions was not reported in any previous studies. So, we hypothesized that this $K_i$ loss in AA cells under isotonic conditions could be the result of an imbalance in intracellular ion concentrations caused by the permanently activated KCC3AA.

According to our hypothesis, doxycycline induces a Cl-dependent $K_i$ loss through KCC matched by a significantly increased RbCl uptake caused by expression of the KCC3 mutant in HEK-293 cells. Doxycycline may also activate a Na channel such as the epithelial Na channel (ENaC) that transports Na into the cell, thus resulting in increased intracellular Na, leading to depolarization and swelling and consequently opening swelling-activated Cl ($Cl_{swell}$) and voltage-sensitive Ca channels, inducing Ca-activated Ca release from intracellular stores, and opening K and Cl channels (Fig. 13). To test this hypothesis we have used ion replacement studies and several inhibitors of Cl, K and Ca channels (Fig. 14). The results will be discussed in the following sections.
Figure 13. Hypothetical model for doxycycline-induced K loss in KCC3 (AA) mutant cells
Figure 14. Approaches to inhibit $K$ loss. The effectors/inhibitors in the diagram are: NMDG (Na replacement), Furo, furosemide, a KCC and anion channel blocker, DCPIB, a volume-sensitive Cl channel blocker, TEA/Clofilium, inhibitors of Ca-dependent and Ca-independent channels, respectively, RR, Ruthenium Red, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetra acetic acid (BAPTA), ethylene diamine tetra acetic acid (EDTA), Ca chelators, RN-1734, a selective TRPV4 channel blocker.

4.6. Effect of extracellular $K$ ($K_o$) on $K_i$ loss in Na medium

According to the Nernst equation, under physiological conditions, even a small rise in $K_o$ leads to a more positive membrane potential and may result in depolarization. So, decrease or complete removal of $K_o$ may lead to hyperpolarization and maintenance of $K_i$. To test this hypothesis, we have used 10 mM Rb, 5 mM K and 0 mM K/Rb flux media maintaining other components constant and the results are plotted in (Fig. 15). Results indicate inhibition of Cl-dependent $K_i$ loss in 5 mM K and 0 mM K/Rb media but no effect on $K_i$ loss in 10 mM Rb. The possible explanation for this inhibition of $K_i$ loss in 5 mM K medium is bidirectional transport of K through KCC i.e. equal K influx and
efflux that, given the existent gradients, results in unchanged $K_i$ whereas KCC inhibition by removal of extracellular K/Rb leads to inhibition of Cl-dependent $K_i$ loss in 0 mM K/Rb medium which is not consistent with the loaded form of the carrier being rate limiting in sheep red cells [12].

![Figure 15. Effect of extracellular K ($K_o$) on $K_i$ in Na medium.](image)

$K_i$ in Cl with no inhibitors (Cl); ouabain (ClO); ouabain + bumetanide (ClOB); and in sulfamate (S) with ouabain + bumetanide (SOB). Flux time, 5 min. n=12; values are means ± S.E. Details are discussed in the text.

### 4.7. Effect of extracellular K ($K_o$) on $K_i$ loss in Na-free medium

According to our hypothesis, doxycycline induces an increase in intracellular Na by opening a Na channel, which may lead to depolarization and $K_i$ loss. Replacing $Na_o$ with NMDG may preclude the Na-induced depolarization and consequently, the $K_i$ loss. So, we have conducted experiments in NMDG under the same conditions described in the former section, i.e. 0 mM K/Rb, 5 mM K and 10 mM Rb flux media. In contrast to expectations, the results were essentially no different from those in Na medium, indicating either no or a partial role of $Na_o$ on doxycycline-induced $K_i$ loss (Fig.16). Furthermore, inhibition of doxycycline-induced Cl-dependent $K_i$ loss even in Na-free media at 0 mM K/Rb and 5 mM K confirmed the role of KCC in this process.
Effect of extracellular K ($K_o$) on $K_i$ in Na-free medium.

Figure 16. Effect of extracellular K ($K_o$) on $K_i$ in Na-free medium. Na was replaced with NMDG. $K_i$ in Cl, with no inhibitors (Cl); ouabain (ClO); ouabain + bumetanide (ClOB); and sulfamate (S) with ouabain + bumetanide (SOB). Flux time, 5 min. n=6; values are means ± S.E. Details are discussed in text.

4.8. Effect of furosemide on Cl-dependent $K_i$ loss

To further investigate the role of KCC in doxycycline-induced $K_i$ loss, we have used 2 mM furosemide, an inhibitor of both NKCC, and KCC [43]. Furosemide, when used only during doxycycline induction had no effect on $K_i$ loss but when used during both preincubation and flux inhibited doxycycline-induced Cl-dependent $K_i$ loss although no effect was observed on Cl-independent $K_i$ loss (Fig.17). As expected, furosemide also reduced RbCl uptake through KCC to baseline levels. In conclusion, the ion replacement studies and those with furosemide confirmed that doxycycline induced a Cl-dependent $K_i$ loss through KCC.
Figure 17. Effect of 2 mM furosemide on $K_i$ loss (K) and Rb influx (Rb). CLO and CLOB refer to $K_i$ after flux in Cl with ouabain, or ouabain + bumetanide, respectively. SOB refers to $K_i$ after flux in S with ouabain + bumetanide. D, doxycycline; furosemide; P, preincubation; fl, flux. n = 9; values are means ± S.E. Details are discussed in the text.

4.9. Effect of TEA on $K_i$ loss

TEA, a blocker of Ca-dependent K channels inhibits the big conductance K channel (BK) at 2 mM and non-selectively other K channels at 10 mM. In addition, TEA inhibited $K_i$ loss in several studies (9, 15). To block the massive doxycycline-induced K loss in AA cells, we used TEA at 2 mM during doxycycline induction alone, during induction and preincubation, or during induction, preincubation and flux. No effect of TEA at 2 mM was observed on $K_i$ loss and Rb influx under any of the tested conditions implying that the BK channel was not responsible for the large $K_i$ loss from AA cells (Fig.18).
Figure 18. Effect of TEA on Ki loss (K) and Rb influx (Rb). ClO and ClOB refer to K<sub>i</sub> after flux in Cl with ouabain, or ouabain + bumetanide, respectively. SOB refers to K<sub>i</sub> after flux in S with ouabain + bumetanide. D, doxycycline; P, preincubation; fl, flux. n = 6; values are means ± S.E. Details discussed in the text.

4.10. Effect of DCPIB on K<sub>i</sub> loss

DCPIB, a volume-sensitive Cl channel blocker, is known to inhibit K<sub>i</sub> loss through IK channels in human lens epithelial cells [49]. DCPIB (50 µM) was used during either flux alone, pre-incubation and flux, or doxycycline induction, preincubation and flux. Complete inhibition of K<sub>i</sub> loss was achieved in the latter condition where K<sub>i</sub> was maintained at the control level. However, DCPIB also inhibited KCC by 50% (Fig. 19)
Figure 19. Effect of DCPIB on $K_i$ loss (K) and Rb influx (Rb). ClO and ClOB refer to $K_i$ after flux in Cl with ouabain, or ouabain + bumetanide, respectively. SOB refers to $K_i$ after flux in S with ouabain + bumetanide. D, doxycycline; P, preincubation; fl, flux. n = 6; values are means ± S.E. Details are discussed in the text.
5. Discussion

A recent study by Rinehart et al. [22] revealed the functional importance of two C-terminal threonine (T) phosphorylation sites; T991 and T1048 in KCC3 regulation. By assessing the increased Rb influx, they demonstrated that replacement of these two amino acids at 991 and 1048 with alanine leads to permanent activation of KCC3 under isotonic conditions. In this study, we investigated the importance of KCC3 and its two functionally important phosphorylation sites T991 and T1048 in K\textsubscript{i} homeostasis, in a human epithelial cell line.

By performing Rb influx studies and measuring K\textsubscript{i} in WT- and AA-transfected HEK-293 cells, we have investigated the perturbed cellular K homeostasis in AA cells. In WT cells, neither KCC nor NKCC activity were affected by 16 h doxycycline induction whereas in AA cells, KCC increased by 25 fold, which is consistent with previous findings. NKCC was inhibited completely and negative (see Fig.6). This negative NKCC in AA cells needs to be further explored.

In AA cells, doxycycline induction lead to a significant K\textsubscript{i} loss (> 90 %) in Cl, and 50 % of this loss was recovered in Cl-free medium, an effect not seen in WT cells, indicating that permanent activation of KCC3 altered K homeostasis. Partial inhibition of K\textsubscript{i} loss in sulfamate medium shows that it has two components, a Cl-dependent and an unidentified Cl-independent K\textsubscript{i} loss. The doxycycline-induced Cl-dependent K loss was
inhibited by replacing extracellular Rb with K, and by 2 mM furosemide (Fig. 12, 13, 14). This finding means that Kᵢ loss occurred through KCC mediated K efflux.

Presence of numerous endogenous voltage-dependent Na, K, Ca and Cl channels, which can generate significant inward or outward currents has been reported for HEK-293 cells in several previous studies (2, 4, 31, 51, 53). So, we attempted to inhibit the doxycycline-induced Cl-independent Kᵢ loss by changing extracellular ion concentrations by ion replacement studies and by various K, Ca and Cl channel inhibitors. Changes in extracellular Na and K did not have any effect on the doxycycline-induced Cl-independent Kᵢ loss. The Ca-dependent K channel blocker TEA (2 mM), reported to block the big conductance (BK) channels in several studies, failed to inhibit the Cl-independent Kᵢ loss, excluding the role of BK channels in AA Kᵢ loss. Clophilium tosylate (100 µM) another Ca-independent K channel blocker had no effect on Kᵢ loss when used either during doxycycline induction alone or in addition to pre-incubation and flux (data not shown). Our experiments with Ca chelators such as EDTA, BAPTA and TRPV4 channel blockers like RN-1734 are inconclusive. DCPIB (50 µM) a volume-sensitive Cl channel blocker when used during (doxycycline + pre-incubation + flux) completely inhibited the Kᵢ loss. However, partial inhibition of KCC by DCPIB prevented us from concluding the role of volume-sensitive Cl channels in Kᵢ loss.

In conclusion, expression of permanently activated KCC3 into HEK-293 cells under doxycycline control results in altered intracellular ion concentrations leading to Kᵢ loss through Cl-dependent and Cl-independent pathways, indicating the importance of KCC3 in cellular K homeostasis. In addition, the Cl-independent K loss is insensitive to
TEA (2 mM), furosemide (2 mM) and to changes in $K_o$ and $Na_o$ concentrations but sensitive to DCPIB (50 µM).
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7. References


cotransporters KCC3 causes deafness, neuro-degeneration and reduced seizure threshold. EMBO.J. 2003; 22(20):5422-34.


