BICARBONATE TRANSPORT IN THE CHICK RENAL PROXIMAL TUBULE

NBC1 AS THE BASOLATERAL TRANSPORTER

by

Kahina Ghanem

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ABSTRACT

Bicarbonate (HCO_3^-) is an important component of acid/base regulation. Therefore, it is crucial to understand the mechanism of bicarbonate reabsorption in the kidney proximal tubule (PT). In mammals the reabsorption mechanism is well understood, but evidence suggests that birds may use a different mechanism. We hypothesize that in the avian PT HCO3- crosses the luminal membrane in ionic form, rather than as CO2, and that HCO3- ions then leave the basolateral side via an NBC1-like transporter, as in the mammalian PT. The experiments used primary cell cultures of chick PT and electrophysiological studies on these monolayers to measure currents (ISC) associated with ion transport. Monolayers were first stimulated with 1µM forskolin, which activated a chloride secretory current in the avian PT. This was followed by basolateral application of 100 µM DIDS, an inhibitor of NBC1. With bicarbonate in the bathing solution DIDS caused an increase in ISC of 6.25 + 1.565 µAmps/cm2 (n = 6), but only 2.30 + 0.58 µAmps/cm2 (n = 5) in the absence of bicarbonate. The increased ISC is consistent with inhibition of electrogenic transport of HCO3- ions via the NBC1 transporter, and the decreased effect of DIDS in the nominal absence of bicarbonate further supports this. Additional studies support both mRNA expression and NBC1 protein expression (western blotting) in chick PT cultures and native tissue. Experiments are underway to test for an alternative apical transporter

Chapter 1

INTRODUCTION

1. Background:

In many organisms, bicarbonate (HCO_3^-) is the major extracellular buffer that maintains the blood pH at a homeostatic value. Bicarbonate acts by reversibly binding protons (H^+) released by various acids produced by metabolism following the reaction $HCO_3^- + H^+ \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_2$. When the concentration of H^+ increases in the plasma, a state called acidosis, more H^+ bind to HCO_3^-, minimizing the change in blood pH. Conversely, when the concentration of H^+ decreases in the plasma, a state called alkalosis, more protons are released from the H_2CO_3 complex thus stabilizing the blood pH. The levels of bicarbonate in the blood are in turn regulated by the kidney. Accordingly, more bicarbonate is excreted to raise H^+ concentration in the plasma and more is produced and reabsorbed to lower H^+ concentration. In mammals, about 80% of the filtered bicarbonate is reabsorbed by the renal proximal tubule (PT) segment of the nephron [1]. The mechanism by which this happens is very well understood. Figure 1 shows the major players and steps that take place during bicarbonate reabsorption in the mammalian PT. In the lumen of the PT, the filtered bicarbonate reacts with protons secreted by the Na/H exchanger (NHE3) or the H^+ ATPase pump and forms H_2CO_3, which then dissociates into H_2O and CO_2 according to the reaction mentioned earlier. In order to speed up the breakdown of H_2CO_3 the reaction is catalyzed by a membrane bound enzyme carbonic anhydrase (CA IV). The resulting CO_2 diffuses through the PT apical membrane via the gas channels aquaporin

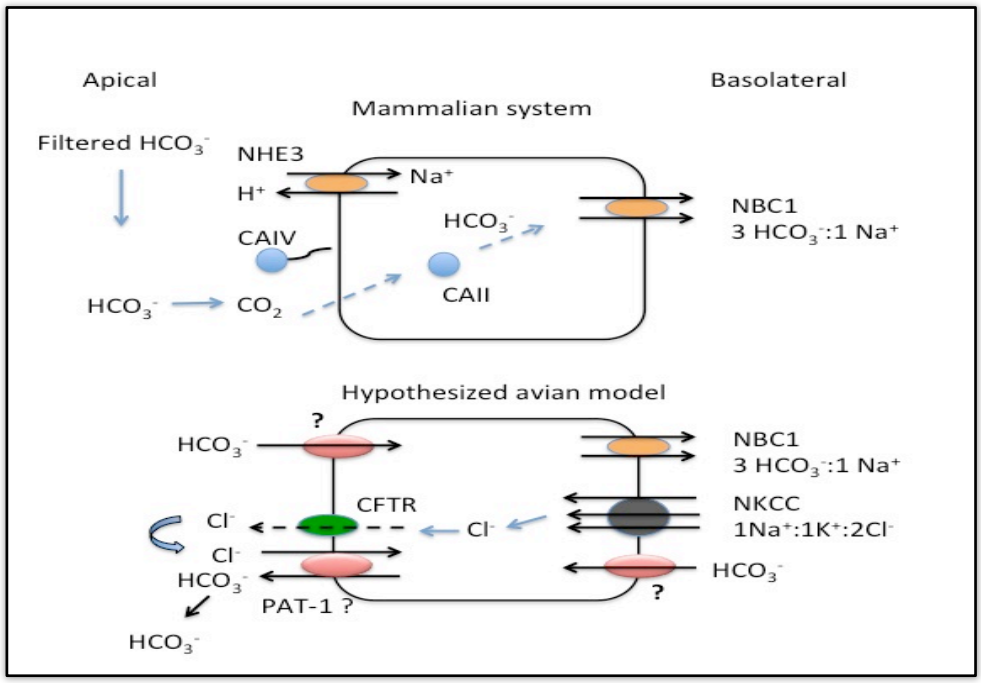

Figure 1: Comparison between mammalian and hypothesized avian model of bicarbonate reabsorption in the proximal tubule.

In mammals (top diagram) the membrane bound enzyme carbonic anhydrase IV converts HCO_3^- into CO_2, which then diffuses into the PT cell where it is converted back into HCO_3^- by the cytoplasmic isoform CA II. However, in birds (lower diagram) no CA IV is detected, implying that HCO_3^- might be transported as an ion via an anion exchanger or other carrier (shown as a question mark). On the basolateral side HCO_3^- is hypothesized to be transported by the cotransporter NBC1 in both mammals and birds. The avian model also shows components of the PTH-activated Cl^- secretory process, which may play a role in reversing the direction of HCO_3^- transport when stimulated by activating an anion exchanger called PAT1.

1[1]. Once inside the PT cell, CO_2 is converted back into bicarbonate by a cytoplasmic carbonic anhydrase isoform (CA II). Bicarbonate is then transported back to the blood by a basolateral electrogenic Na-HCO_3^- cotransporter (NBC1), while H^+ are recycled back to the lumen [1]. PT reabsorption of bicarbonate can be blocked by the calcitropic parathyroid hormone (PTH). This is accomplished by the inactivation by phosphorylation of Na-H exchanger NHE-3 [2] and results in blocking the titration of bicarbonate. As a result bicarbonate reabsorption is prevented at the PT level. Instead, bicarbonate reabsorption is shifted to the distal tubule where its presence in high concentration enhances the reabsorption of calcium ions (Ca^{2+}) [1, 3, 4].

Contrary to mammals, the exact mechanisms of bicarbonate reabsorption in the avian PT remain unresolved. A number of morphological and physiological differences are observed in the avian kidney. For instance, in the avian kidney, two main types of nephrons are distinguished: 1. “mammalian type” (MT) nephrons with long convoluted proximal and distal tubules and long loops of Henle, which run parallel to the collecting duct and 2. a majority of “reptilian type” (RT) nephrons, smaller in size, with simple proximal and distal tubules, no loops of Henle, and emptying at right angles of the collecting duct [5,6]. Figure 2. shows the avian kidney morphology with the two types of nephrons and their relative positions. The avian kidney also possesses a functional renal portal blood supply, by which venous blood from the posterior body mixes with post-glomerular arterial blood in the kidney [5]. These morphological differences suggest that there might be functional differences as well. For example, histochemical staining experiments were not able to detect CA activity in the proximal tubule of the European Starling kidney [7] as well as other avian species [8, 9, 10]. However, when the same staining procedure was performed in

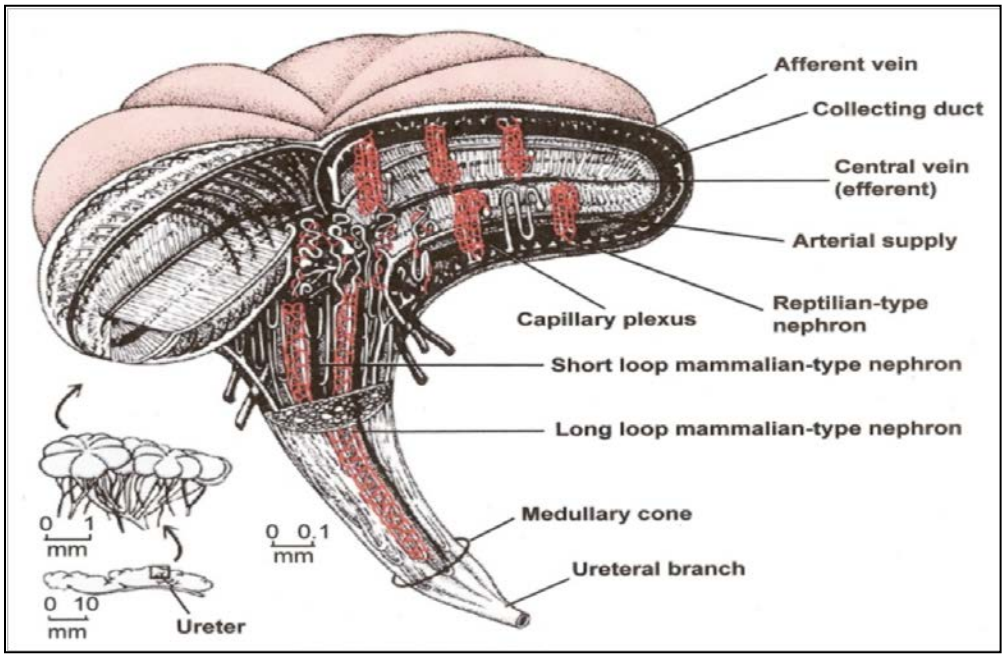

Figure 2: 3D drawing of an avian medullary cone.

At the lower left a drawing of a whole kidney is shown. Above the whole kidney, part of the kidney is shown at a higher magnification. At an even higher magnification an avian medullary cone is showing the different types of nephrons found in the avian kidney and their relative positions. The reptilian type nephrons are smaller and positioned at right angles from the colleting duct compared to the looped mammalian type nephrons (modified from Dantzler, 1980 [6]).

mouse and rabbit kidney, CA activity was detected [11]. Figure 3 shows pictures of the staining in the different organisms mentioned above. Another interesting difference is that in chick PT primary cell cultures, PTH was shown to induce Cl^- secretion through the activation of the Cl^- channel cystic fibrosis transmembrane conductance regulator or CFTR [12]. This was a puzzling observation, since PTH had never been physiologically associated with chloride ion regulation.

However, the overall effect of PTH on bicarbonate in birds remains the same, namely that bicarbonate accumulates in the urine [13]. A possible explanation for this is that PTH in birds reverses the direction of HCO_3^- ion transport by PT cells, and uses the CFTR-mediated Cl^- ion secretion as a mechanism to drive apical transport of HCO_3^- ions into the lumen.

The absence of CA in PT, and the fact that PTH has different effects on the PT cells, yet results in the same outcome, suggests that bicarbonate might be handled differently in the avian kidney in general and in the PT specifically. The hypothesis in this study is that in the avian proximal tubule bicarbonate is transported as an ion, as opposed to diffusion of CO_2 gas. In this study we suggest that bicarbonate crosses the apical membrane via an anion transporter, possibly an exchanger from the SLC26A family or a homolog of the NBC1 transporter, and that bicarbonate then crosses the basolateral membrane through another Na-HCO_3 cotransporter like NBC1. Figure 1 shows the hypothesiszed model of bicarbonate reabsorption and secretion. In order to thoroughly answer the question, the functional, genetic and protein expression of the transporters of interest will be tested. The experimental strategy was to first look at the basolateral side, in order to characterize the basolateral transporter and then to move on to the apical side and characterize the apical transporter. Thus, most of the data

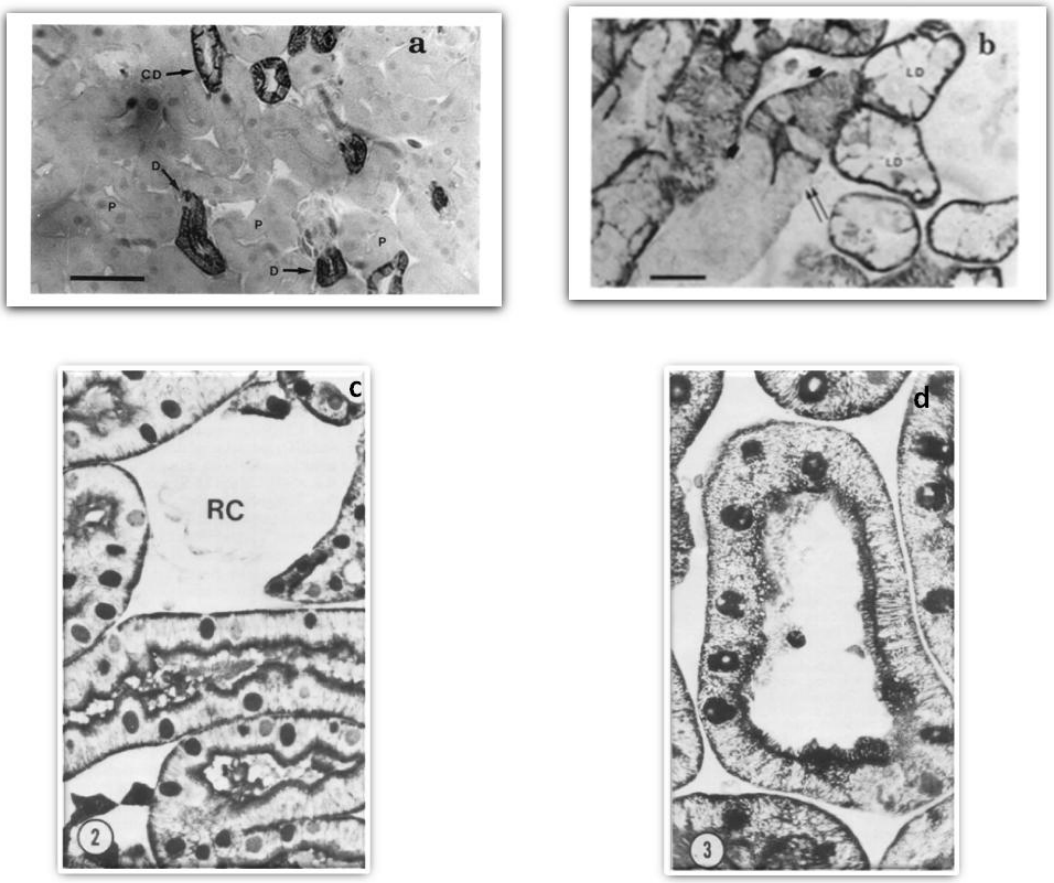

Figure 3: Avian and mammalian carbonic anhydrase activity.

(a and b) histochemical staining showing CA activity detected enzyme in distal tubule but not in proximal tubule of European starling. (Image retrieved from Laverty et al, 1991, [7]). (c and d) the same histochemical staining method showing CA activity in proximal tubule of rabbit (c) and mouse (d). (Image from Dobyan at al, 1982 [11])

presented in this thesis will address the potential expression and function of a basolateral NBC1 homolog in the avian kidney.

2. Na-HCO3 cotransporter NBC1:

NBC is an electrogenic Na-HCO_3 cotransporter responsible for the reabsorption and secretion of bicarbonate in many epithelia. Isoforms of NBC have been detected in the kidney, pancreas, brain, small intestine, colon, epididymis, eye, heart, liver, salivary glands, stomach, and testis [14]. NBC1 is the isoform responsible for the reabsoption of bicarbonate in the kidney. It is generally referred to as kNBC1. It is encoded by the gene SLC4A4 located at 4q21 [15, 16]. Interestingly, from a different promoter, SLC4A4 also encodes the pancreas isoform pNBC1 [14, 17], which is responsible for the secretion of bicarbonate into the pancreatic duct. Moreover, it was shown that when kidney cell lines were transfected with pNBC1, this latter performed kNBC1 reabsorptive function, suggesting that NBC1 function is closely related to the environment it is in [18], possibly involving accessory proteins [14]. The human kNBC was found to have a sequence of 1035 amino acids and a predicted size of 116 kDa [19, 20, 21, 22], with intracellular N- and C-termini [14]. Figure 4. Shows the NBC1 was shown to be activated by Ca-dependent protein kinase, angiotensin II, and cholinergic stimulants, all of which lower the intracellular concentration of cAMP [23, 24, 25, 26,]. It is also known that NBC1 can be inhibited by stilbenes such as 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) [27]. In the pNBC1 predicted protein, the first 41 amino acids on the N-terminus are

replaced by a different sequence of 85 amino acids. So instead, pNBC1 encodes for 1079 amino acids and predicts a protein of 120 kDa with possible different phosphorylation sites [14, 28]. pNBC1 is also known to be activated by the cystic fibriosis transmembrane conductance regulator CFTR [29]. The stoichiomerty of kNBC1 is generally accepted to be 1 Na^+: 3HCO_3^- in the proximal tubule [30, 31]. However, it is hard to tell the exact form under which bicarbonate is cotransported as the ratios 1Na^+:3 HCO_3^-, 1Na^+ : 2 HCO_3^- :1OH^- , and 1Na^+:1 HCO_3^-: 1CO_3^{2-} are thermodynamically equivalent [29]. It should also be noted that pNBC1 stoichiometry was found to be 1 Na^+: 2HCO_3^-. The first aim of this proposal was to obtain functional evidence for NBC1 in the avian renal proximal tubule.

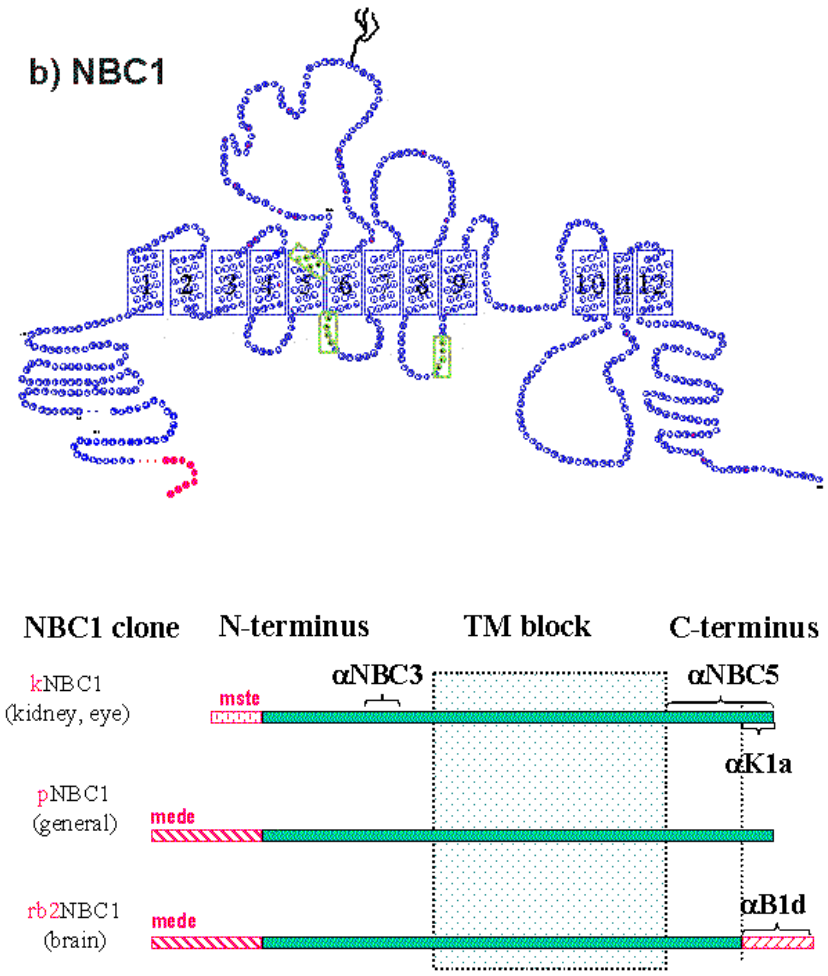

Figure 4: Working membrane topology model of NBC.

NBC1 is embedded in the basolateral membrane of the kidney proximal tubule cell. NBC1 is predicted to have twelve transmemebrane spans with cytosolic C and N Termini. The green colored boxes represent predicted binding sites of the inhibitor DIDS (Image from Romero, 2001 [14])

The figure below shows the three NBC1 isoforms. pNBC1 and kNBC1 have identical C-termini but differ in their N-termini.

Chapter 2

MATERIALS AND METHODS

Reagents, supplies and animals:

Growth media and supplements, collagen I, Percoll, forskolin and DIDS were obtained from SigmaAldrich (St. Louiis, MO). Collagenase A was obtained from Roche Applied Science (Indianapolis, IN). The growth medium was serum-free DMEM-Ham's F-12 (1:1). The NBC1 antibody was purchased from EMD Millipore (Billerica, MA) and polyacrylamide gels from Life Technologies (Grand Island, NY). Primers for chicken NBC1 were designed and ordered from Integrated DNA Technologies (Coralville, IA). Chick PT monolayers for electrophysiology were grown on Corning Transwell PET membrane filter inserts with 0.4 μm pore size and 0.33 cm^2 surface area obtained from Fisher Scientific). Chicks were obtained from the University of Delaware Poultry Unit where they hatched and raised until needed. They were killed by cervical dislocation in accordance with the protocol approved by the University's Institutional Animal Care and Use.

Cell culture:

Following the protocol described in Sutterlin and Laverty (1998) [32], white Leghorn chicks (*Gallus gallus*) one to two weeks old were sacrificed by cervical dislocation one at a time and their kidneys were aseptically harvested, washed in ice cold Hank's balanced salt solution (HBSS), and cleared of any visible blood vessels and connective tissue. The kidney pieces were then cut with dissecting scissors into smaller pieces and washed two more times with ice cold HBSS and were kept on

HBSS on ice until all the kidneys were harvested. The smaller pieces were then minced with a sterile scalpel and transferred to 10 ml growth medium containing 1.0 mg/ml collagenase A. The mixture was then incubated and gently rocked for 30 minutes at 37°C with 5% CO_2 and 95% O_2 air flow. After incubation, in order to further separate individual tubules, the mixture was triturated for 2 minutes using a 10 ml pipette. The mixture was then passed through a stainless steel sieve. The collected filtrate was washed of any excess blood cells and centrifuged in HBSS at 500 g. This step was repeated 3 times. After the final wash, the pellet was resuspended in a 1:1 Percoll-2X Krebs-Hensleit buffer containing 240 mM NaCl, 8 mM KCl, 2 mM KH_2PO_4, 30 mM $NaHCO_3$, 2.4 mM $CaCl_2$, 2.4 mM $MgSO_4$, 10 mM glucose, and 20 mM HEPES, which forms a density gradient. The suspension was centrifuged at 17,500 g for 30 min at a temperature between 5 and 10 °C. The proximal tubule fragments formed an easily recognizable high density band at the bottom of the tube. The PT band was extracted using a sterile Pasteur glass pipette and was washed and centrifuged in HBSS at 500 g. This step was repeated three times, and a fourth time with growth medium. The final pellet was then resuspended in 4-5 ml growth medium and seeded in 4 to 8 Transwells precoated with collagen, or in T25 flasks for total RNA and protein extraction. The cells were then incubated at 37 °C with 5% CO_2 and 95% O_2 air flow and fed every two days. The PT fragments eventually attached to the porous membrane and formed a monolayer that reached confluence after 10-14 days. Confluence of the PT primary culture was monitored by measuring the electrical resistance in situ by epethila volt-ohmmeter EVOM (World Precision Instruments, Sarasota, FL). Only cultures with resistance higher than 300 Ω were used in the electrophysiology experiments.

Electrophysiology:

Proximal tubule (PT) cells were grown to confluence on filter inserts (Transwells) and were mounted on modified Ussing chambers. The edge of the Transwell was sealed with a rubber "O" ring and the PT monolayer formed a complete barrier between the apical and basolateral half chambers (see Figure electrophysiology apparatus). The two reservoirs of the half chambers were filled with a transport buffer that was heated to 37°C and circulated and bubbled with either 95% O_2/5% CO_2 or with 100% O_2. Two types of buffers were used in the electrophysiology experiments, a normal transport buffer (NTB) containing 130 mM NaCl, 4 mM KCl, 1.3 mMCaCl2.2H2O, 1 mM MgSO4.7H2O, 5 mM HEPES, and 25 mM $NaHCO_3$ and a pH of 7.54 that was circulated with 5% CO_2 and 95 % O_2 and a bicarbonate free buffer containing 155 mM NaCl, 4 mM KCl, 1.3 mM CaCl2.2H2O, MgSO4.7H_2O, and 10 mM HEPES and a pH of 7.54. When the bicarbonate free buffer was used it was circulated with 100% O_2. Before each experiment, the fluid resistance was corrected before insertion of the monolayers. The potential difference (PD) of the monolayer was measured by coupling the apical and basolateral reservoirs with a pair of calomel half cells through two Ringer-agar bridges. The PD was continuously clamped by an automatic two channel voltage clamp (EC825, Warner Instruments). The apical and basolateral reservoirs were connected through two Ringer-agar bridges to two Ag/AgCl wires, which sent the short circuit current (I_{sc}). The I_{sc} was recorded on a strip-chart recorder and a data acquisition system (PowerLab, AD Instruments). Transepithelial resistance was monitored by a voltage change of 1mV for 2 seconds every 5 minutes which caused a current deflection.

For each experiment two monolayers from the same culture were selected. One monolayer was bathed in a NTB buffer while the other one was bathed in a HCO_3^- -free buffer. After the baseline current stabilized which, usually took 5 minutes, 5 mM glucose was added to activate a sodium-glucose cotransporter (marker for proximal tubule cells), followed by 1µM of the adenylyl cyclase activator, forskolin, and 100µM basolateral NBC1 inhibitor, DIDS, with 15 minutes intervals between each addition. Monolayers for different experiments came from different cultures. Forskolin has been previously shown (Laverty et al, 2012) to activate apical CFTR and overall net Cl^- secretion by the chick proximal tubule epithelium (positive current) [12].

Reverse-Transcriptase PCR (RT-PCR):

Total RNA was extracted from primary PT cells cultured on T25 flasks or from native PT fragments isolated from the chick kidney according to the previously mentioned procedure. A PureLink Micro-to-Midi Total RNA Purification System Kit (Invitrogen) was used for the extraction of RNA. The RNA samples were DNase treated and quantitative as well as qualitative analysis were performed using Eppendorf Biophotometer. cDNA was synthesized and amplified using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen) with a Techne thermocycler. The negative controls (minus RT) were kept on ice during the programmed reverse transcription step of cDNA synthesis.

Primers for chicken NBC1 were designed using a bioinformatics approach. Using the “homologene” tool on the National Center for Biotechnology Information (NCBI) website, a conserved NBC1 sequence was identified (Accession number XM_420603.3). NBC1 primers were then designed using the PrimerQuest software

from Integrated DNA Technologies (IDT, Inc, CoralVille, IA, USA) and purchased from IDT. The primers sequences were: forward: TCTGCGTCATGTCCCGCTGC , reverse: GTAAGGGTGGAGCGGGGAGC reverse. The predicted product length was 346 bp. The RT-PCR products were run on 1.5 % agarose gel and visualized with ethidium bromide.

Western Blotting:

Total cell lysates were prepared from cultured PT cells and from native PT fragments. The lysis buffer was composed of 300 µl H_2O, 50 µl of 10x RIPA lysis buffer (Cell Signaling Technology), 50 µl 10X CompleteMini protease inhibitor cocktail (Roche Applied Science), 1µl PMSF, 1µl aprotinin, 1µl leupeptin, 50 µl 1% sodium dodecyl sulfate (SDS), and 50 µl 10 % Triton X-100. Three hundred microliters of this was added to a T25 flask of PT cells or to 50-100 mg of native tissue (PT fragments from the Percoll gradient) and kept on ice for 2 min. The tissue was then scraped off and homogenized using a syringe. The mixture was then centrifuged for 1 min to remove any cellular debris that collected in a pellet at the bottom of the tube. Lysates were kept in -70°C freezer until needed. The loading buffer was prepared by adding 25 µl of 2-mercaptoethanol to 500 µl Laemmli Sample buffer (BIO-RAD). 25µL of the loading buffer was added to 25 µl of each sample and transferred into a 37°C water bath for 30 min. 20 µl of the different samples were loaded into a 4-15% polyacrylamide gel (Life Technologies) and electrophoresed at 150V for 60 min, then transferred to a nitrocellulose paper. The nitrocellulose paper (blot) was then blocked with 5% milk solution for 1 h and then treated with a 1:500 dilution of rabbit primary antibody overnight (EMD-Millipore) . After a series of two washes with TBS and TBST, the nitrocellulose paper was treated with a 1:30,000

dilution of anti-rabbit secondary antibody (Jackson Labs) and Strep Tactin HRP (BIO-RAD) for 75-90 min.

Data analysis and statistics:

Data was expressed in means ± SEM. Significance was assessed by using a paired student t-test.

Chapter 3

RESULTS

1. Electrophysiology:

Normal transport buffer (NTB):

Electrophysiology experiments were carried out to show the functional expression of the cotransporter, NBC1. By convention, positive charges transported across the epithelial cell layer from the apical to the basolateral side are equivalent to negative charges transported across the epithelial cell layer from the basolateral to the apical side and are referred to as positive current. By contrast, positive charges transported across the epithelium from the basolateral side to the lumen and negative charges transported from the lumen to the basolateral side, are referred to as negative current. In the normal transport buffer (NTB) experiments, 6 monolayers from 6 different cultures were bathed in NTB. Even though the baseline values between individual monolayers were variable, ranging from 5.60 μAmp/cm² for the lowest and 18.88 μAmp/cm² for the highest, the addition of 5mM glucose consistently caused an increase in the short circuit current (I_{sc}) of 2.56 ± 0.63 μAmp/cm² in average. Similarly, the addition of 1μM forskolin provoked a consistent increase in current larger than that of glucose and characterized by an overshoot that lasted in average 1 min, followed by a stabilization of the I_{sc} at an average value of 13.40 ±1.51 μAmp/cm². Although forskolin-activated current responses have been shown to be largely due to CFTR-mediated Cl^- secretion (positive current), the overall current may also include a component of HCO_3^- ion transport (negative current). Addition of

100μM DIDS to the forskolin-treated monolayers caused a consistent increase in current of 6.25 ± 1.55 μAmp/cm², which is consistent with inhibition of a negative component of the I_{SC}. Figure 5.a. shows the time course of the average I_{sc} values of the 6 monolayers in NTB with error bars.

For the bicarbonate free buffer, 5 monolayers from 5 different cultures were used. A variation in the baseline current was also observed between individual monolayers ranging from -2.05 μAmp/cm² to 12.60 μAmp/cm². Figure 5.b. shows the time course of the average I_{sc} values of the 5 monolayers in a HCO_3^--free buffer. While there was no significant difference in the glucose and forskolin responses compared to the NTB experiments, 2.00 ± 0.49 μAmp/cm² and 11.03 ± 1.32 μAmp/cm² respectively, the DIDS response was smaller with an increase in current of 2.30 ± 0.58 μAmp/cm². Furthermore, when a paired t-test was performed between the I_{sc} before and after addition of DIDS, it was found to be not significant in the HCO_3^- buffer ($P < 0.05$). Figure 6 shows a comparison of specific time points after each treatment.

RT-PCR and Western blotting:

RT-PCR experiments, designed to show the genetic expression of NBC1, showed the presence of NBC1 mRNA in both cultured PT and native PT. (See Figure 7.). Two bands of the predicted size 346 bp were observed while no bands were observed in the negative control, confirming that the detected bands were derived from mRNA. This experiment was repeated twice with identical results.

Western blot experiments were performed to show the protein expression of NBC1. Protein bands of the predicted size (130 kDa) were detected by the 1° antibody

in both PT cultures and native tissue. In addition, the antibody detected an NBC1 band in OK cell line used as a positive control. (Figure 8.)

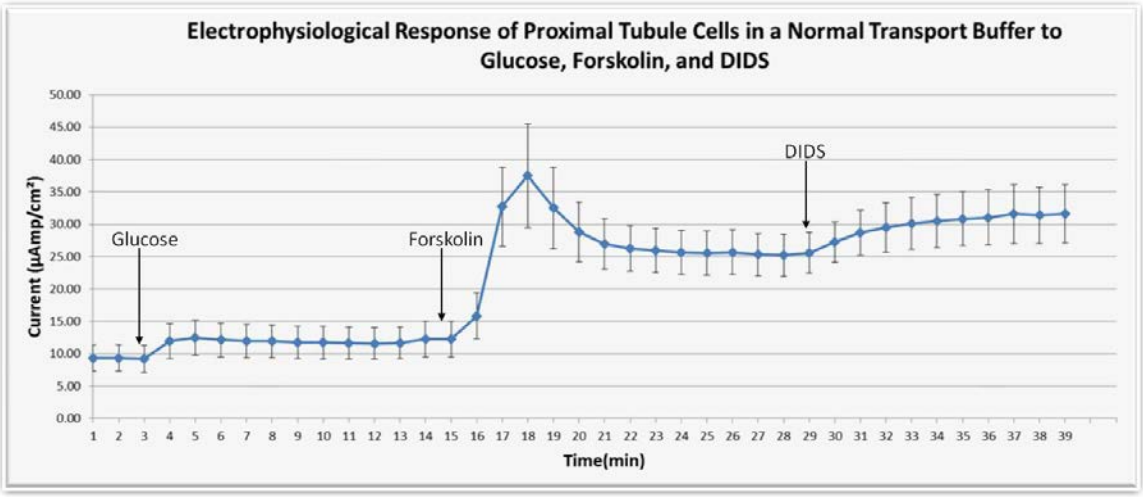

Figure 5.a. Electrophysiological response of proximal tubule cells in a normal transport buffer to glucose, forskolin, and DIDS.

The time course represents the mean values of I_{sc} of 6 monolayers bathed in normal transport buffer (NTB), containing bicarbonate and gassed with 5% CO_2/ 95% O_2. At time = 3min, 5mM glucose was added, time = 15min, 1µM forskolin was added, and at time = 29, 100µM DIDS was added to the basolateral side.

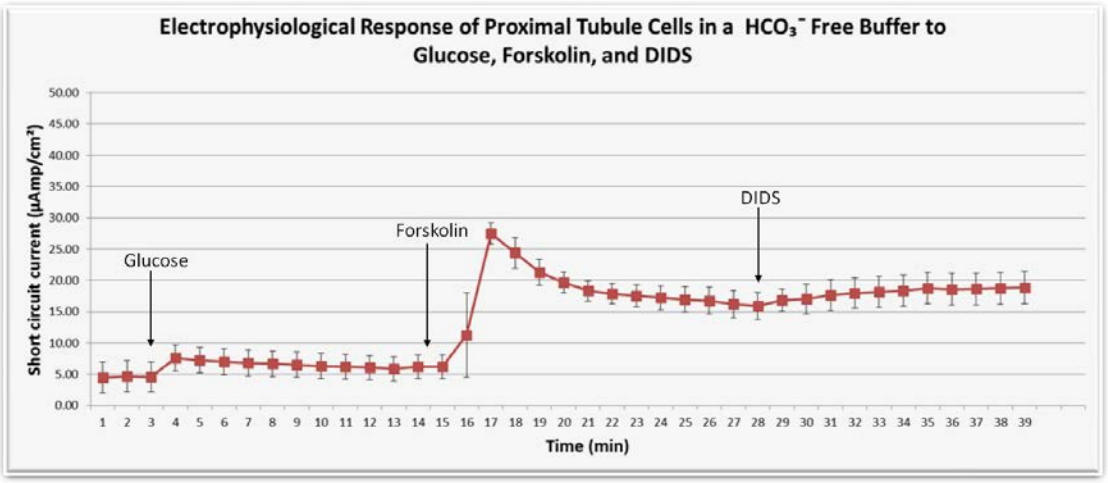

Figure 5.b. Electrophysiological response of proximal tubule cells in a hco_3^- free buffer to glucose, forskolin, and DIDS.

The time course represents the mean values of I_{sc} of 5 monolayers bathed in a bicarbonate free buffer gassd with 100% O_2. At time = 3min, 5mM glucose was added, time = 15min, 1µM forskolin was added, and at time = 28, 100µM DIDS was added to the basolateral side.

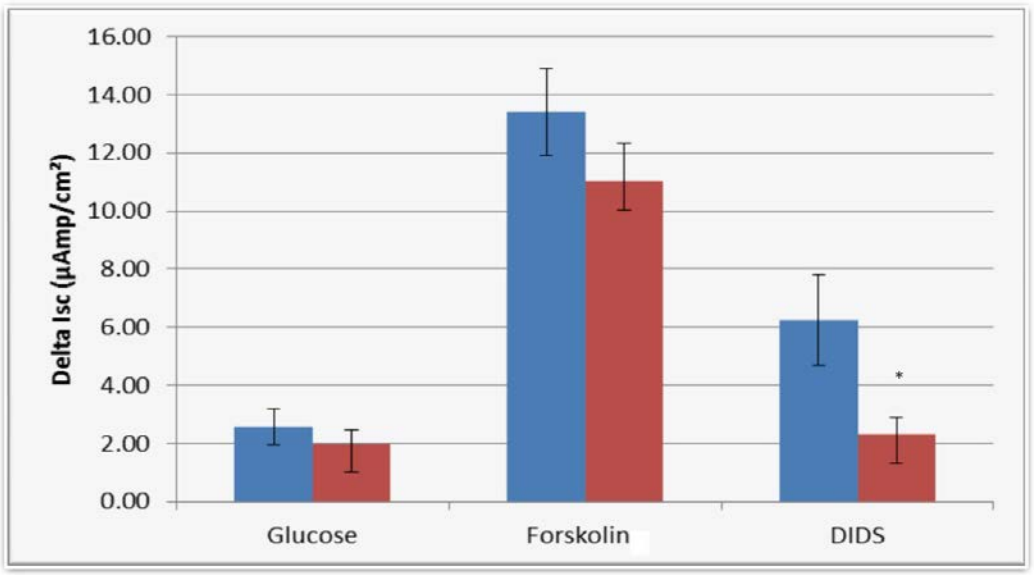

Figure 6: Comparison of time points representative of effects of glucose (5mM), forskolin (1µM), DIDS (100µM) on primary cell cultures of the chick proximal tubule.

Even though the effects of glucose and forskolin are comparable in both buffers, normal transport buffer (blue, n = 6) and a bicarbonate free buffer (red, n = 5), the response to DIDS is different with a much stronger response in the normal transport buffer for both. * The change in I_{sc} between forskolin and DIDS was significant ($P < 0.03$ by Student paired t-test)

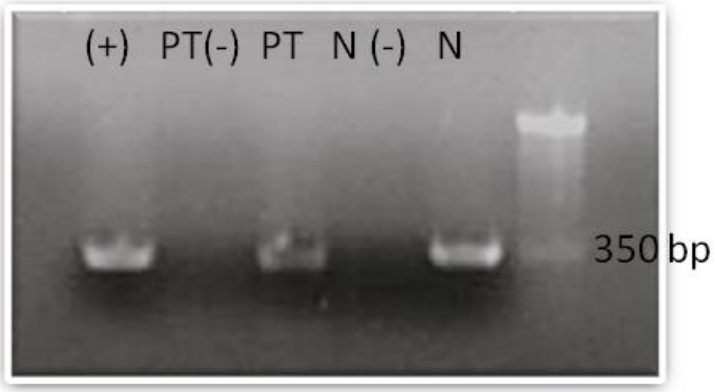

Figure 7: Chick NBC1 RT-PCR.

Specific primers for predicted chick NBC1 were carefully chosen to run the RT-PCR . Total RNA from both native tissue (N) and primary cultures (PT) were used. The RT-PCR revealed the presence of NBC1 mRNA in both sample types at the predicted size of 346 bp. (+) represent GAPDH used as a positive control. PT (-) and N (-) was the PT and native tissue negative control (-RT), respectively.

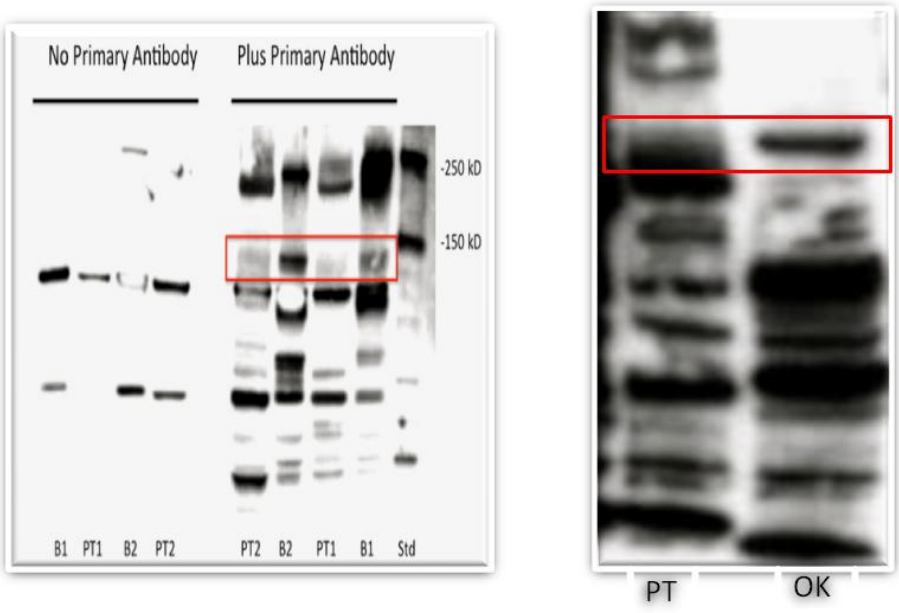

Figure 8: Pictures of NBC1 western blots using a polyclonal NBC1 antibody.

Protein samples were extracted from native PT tissue (B1, B2) and from PT primary cultures (PT, PT1, PT2). Proteins from a mammalian OK cell line culture was used as a positive control. The NBC1 band was observed at 130 kDa in PT cultures, native PT, and OK cells (red boxes). Additional, lower MW bands are also observed in both chick samples and in the positive control. The full blot on the left includes a control half-blot, in which identical samples were electrophoresed and blotted, but were not exposed to the primary NBC1 antibody. Two non-specific bands are routinely seen in chick lysates (approximately 60 and 120 kD).

Chapter 4

DISCUSSION

The results obtained from the RT-PCR, western blotting , and electrophysiology experiments provide evidence for the existence of a basolateral NBC1 like transporter in the avian proximal tubule. The detection of bands in the RT-PCR experiments, at the predicted size, in both native PT and cultured PT provide evidence for the presence of mRNA in the chick PT cells. Similarly, the detection of protein bands in the western blots at the predicted size, in both cultured and native PT tissue is evidence that the NBC1 protein is expressed in the chick PT. In addition, the electrophysiology experiments showed the functional expression of NBC1. In the normal transport buffer (NTB) experiments, the basolateral addition of the inhibitor DIDS caused an increase in the I_{sc} current, which was larger than that of the bicarbonate free buffer experiment. Furthermore, it appeared that the addition of forskolin prior to DIDS amplified this effect. In the NTB experiments, because of the presence of bicarbonate and a physiological pH of 7.54, NBC1 was expected to carry its normal function of bicarbonate transport. The increase in current upon addition of DIDS was expected and can be explained by the inhibition of the negative current resulting from the negatively charged bicarbonate ions being transported across the basolateral membrane via the electrogenic cotransporter NBC1. Removal of bicarbonate in the bicarbonate free buffer experiments, served to show that the increase in current in the NTB experiments, after addition of DIDS, was indeed directly tied to the presence of bicarbonate and not to other ions. In the nominal

absence of bicarbonate, the activity of NBC1 was expected to be reduced and no increase in current was predicted upon addition of DIDS. However, it turned out that it was very difficult to have a buffer that was completely free of HCO_3^-. The PT cells being metabolically active, were constantly generating CO_2, a fraction of which was spontaneously hydrated and turned into HCO_3^-. Thus, the observed small increase in current in the HCO_3^- free buffer could be explained by the presence of HCO_3^-, although in smaller concentrations. In addition, DIDS being an impermeant inhibitor [33], and the observed immediate effect after the addition of DIDS offers further evidence that the NBC1 transporter affected was on the basolateral side.

The stimulatory effect of forskolin , which is an adenylyl cyclase activator that causes an increase of intracellular cAMP in the avian PT and activates a Cl^- secretory response, on NBC1was puzzling. Studies have previously found that forskolin had a stimulatory effect on the pNBC1 isoform in the mouse colon cells [34]. However, other studies done on mammalian proximal tubule kNBC1, showed inhibitory dose-dependent effects of cAMP generating substances such as PTH, and cAMP-dependent protein kinase [35]. Even more surprising, subsequent studies done to test the effect of forskolin on pNBC1 transfected into human embryonic kidney (HEK293) cells, showed that forskolin and the presence of cAMP had an inhibitory effect on the transfected pNBC1 [36]. This is evidence that the cell environment in which the NBC1 isoform is expressed affects the stoichiometry and direction of transport. Another study further proved this point by transfecting pNBC1 into mammalian PT cells, and kNBC1 into mammalian collecting duct cells. The stoichiometry of pNBC1

changed from $1Na^+ : 2HCO_3^-$ to $1Na^+ : 3HCO_3^-$ with an accompanying change in direction of transport from secretory to reabsorptive whereas, the kNBC1 stoichiometry changed from $1Na^+ : 3HCO_3^-$ to $1Na^+ : 2HCO_3^-$ with a reversal of transport [18]. The factors affecting the cell environment possibly involve regulatory proteins that are different form a cell type to another. This suggests that there might be a factor or factors, presumably proteins, which are expressed in the avian PT cells, possibly of the same nature as some of the ones expressed in the pancreas in addition to other regulatory proteins found in the mammalian PT.

The presence of CFTR in the avian proximal tubule, not normally found in the mammalian proximal tubule, might be a factor that helps explain the stimulatory effect of forskolin in the avian PT. As mentioned earlier, CFTR is known to have a regulatory effect on pNBC1 in the pancreas and intestine [29]. One way to verify this would be to look for the inositol 1,4,5-trisphosphate (IP3) receptor binding protein IRBIT, which can specifically bind to and activate pNBC1 and CFTR [37, 38], and is also known to coordinate the activities of pNBC1 and CFTR. However, while in the pancreas the direction of HCO_3^- transport is secretory, in the avian PT the increase in current indicates a reabsorptive pathway. This is evidence for the existence of a more complicated setting possibly involving more than one NBC1 isoform.

At the same time, other studies have pointed to the presence of different phosphorylation sites in different NBC1 subtypes that determine differences in regulation. For example, Gross et al, 2001[39], has shown that an increase in cAMP causes the activation of PKA which in turn phosphorylates the Ser^{982} of kNBC1.

transfected into mouse renal proximal tubule cell line deficient in electrogenic sodium bicarbonate cotransport function. This phosphorylation causes transfected kNBC1 to change its stoichiometry from $1Na^+ : 3HCO_3^-$ to $1Na^+ : 2HCO_3^-$,which then changes the direction of transport [39]. This same effect may be happening with PTH in the avian PT, which also affects CFTR to secrete Cl- in order to drive bicarbonate out.

It has also been shown that more than one subtype of NBC1 can be expressed in a given tissue [14, 29]. For example, in the mouse colon, small amounts of kNBC1 are expressed in addition to pNBC1 [40]. This could be also the case in the avian PT where both NBC1 isofroms might be present, one involved in the reabsorption of bicarbonate and the other in the secretion of bicarbonate, depending on the levels of cAMP and the proteins involved in the pathway. Two predicted NBC1 mRNA's associated with two predicted NBC1 isoform sequences have been identified in the chicken database. These two isoforms differ in their C-terminal sequences, suggesting that the possibility of the coexistence of two NBC1 isoforms in the avian PT is not out of the question.

Taken together, the presence of both NBC1 isoforms in the avian proximal tubule with their respective regulatory protein helps explain the differences between mammalian and avian model of bicarbonate regulation.

Future work:

In order to further investigate the functioning and regulation of NBC1 in the avian PT, western blotting experiments using an IRBIT antibody will be performed to detect its presence in cultured and native PT. The functional expression of IRBIT will then be tested by treating PT monolayers with small interfering RNA (siRNA) that

would interfere with the translation of IRBIT. The effect of forskolin and DIDS on NBC1 and CFTR after blocking IRBIT will then be investigated by electrophysiology.

The genetic expression of the different NBC1 isoforms in the chick PT will be investigated by designing primers at the C-termini of the two predicted sequences of the chick NBC1 isoforms and RT-PCR experiments will be performed using PT cell cultures and native tissue. If the mRNA's of the two different isoforms are detected in the avian PT, they will be used to obtain the amino acid sequences of the two isoforms.

Preliminary electrophysiology experiments have suggested that there is a Cl^- dependent HCO_3^- apical transporter that is sensitive to DIDS and affected by forskolin.

In order to study the functional expression of the apical bicarbonate transporter, more electrophysiology experiments will be carried using PT primary cultures grown on Transwells. In order to isolate the apical side of the PT culture, the basolateral side will be permeabilized by adding nystatin, which renders the basolateral side of the PT culture permeable to small monovalent anions and cations such as Na^+, K^+, and Cl^-. This permeabilization facilitates the study of the apical transport by getting rid of basolateral active transport and has been used previously to study the apical function of CFTR in chick PT cultures [12]. Different combinations of Cl^- and HCO_3^- gradients will then be set and the effects forskolin and DIDS under different circumstances will be investigated. It is also not improbable that an NBC1 like cotransporter is expressed on the apical side and serves as the apical bicarbonate transporter in bicarbonate reabsorption. In order to test this, immunostaining using NBC1 specific antibody will be performed on chick kidney sections.

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