Altered intracellular pH regulation and Na⁺/HCO₃⁻ transporter activity in choroid plexus of the cilia defective Tg737orpk mutant mouse

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Abstract

*Tg737*orpk* mice have defects in cilia assembly and develop hydrocephalus in the perinatal period of life. The hydrocephalus is progressive and is thought to be initiated by abnormal ion and water transport across the choroid plexus epithelium. The pathology is further aggravated by the slow and disorganized beating of motile cilia on ependymal cells that contribute to decreased cerebrospinal fluid movement through the ventricles. Previously, we demonstrated that the hydrocephalus phenotype is associated with a marked increase in intracellular cyclic AMP levels in choroid plexus epithelium, which is known to have regulatory effects on ion and fluid movement in many secretory epithelia. To evaluate whether the hydrocephalus in *Tg737*orpk* mutants is associated with defects in ion transport, we compared the steady-state intracellular pH and Na\(^{+}\)-dependent transport activities of isolated choroid plexus epithelium tissue from *Tg737*orpk* mutant and wild-type mice. The data indicate that *Tg737*orpk* mutant choroid plexus epithelium have lower pHi and higher Na\(^{+}\)-dependent HCO\(_3\)\(^{-}\) transport activity compared to wild-type choroid plexus epithelium. In addition, wild-type choroid plexus epithelium could be converted to a mutant phenotype with regard to the activity of Na\(^{+}\)-dependent HCO\(_3\)\(^{-}\) transport by addition of dibutyryl-cAMP and mutant choroid plexus epithelium toward the wild-type phenotype by inhibiting PKA activity with H-89. Together, these data suggest that cilia have an important role in regulating normal physiology of choroid plexus epithelium and that ciliary dysfunction in *Tg737*orpk* mutants disrupts a signaling pathway leading to elevated intracellular cyclic AMP levels and aberrant regulation of pHi and ion transport activity.

Keywords: choroid plexus, cAMP, *Tg737*orpk* mice, cilia, ion transport
Introduction

A growing body of evidence indicates that cilia integrity is required for normal cell function (36). Cilia are complex organelles containing more than 500 peptides involved in their formation and function (22). There are motile and immotile (primary cilia) forms of cilia that have diverse function ranging from fluid and cell movement to sensory perception. Motile cilia are found on epithelial cells in the lung and ependyma lining the brain ventricles, while primary cilia are present on most epithelial and non-epithelial cells of the body.

Cilia are assembled through a process called intraflagellar transport (IFT) which mediates the bidirectional movement of large protein complexes (IFT particles) along the microtubule-based axoneme. Anterograde movement from the base toward the tip of the cilium occurs by a kinesin-II complex and retrograde from the tip back to the base by a dynein driven complex (35). Polaris is one of the IFT particle proteins and is the product of the Tg737 gene. The Tg737 gene is highly conserved with orthologs in C. elegans (OSM-5), Drosophila (NOMPB) and Chlamydomonas (IFT88) (13, 17, 26, 31, 39). Null mutations in Tg737 or its orthologs in other organisms result in the loss of cilia or flagella (26, 48). In mice, Tg737 null mutations result in midgestation lethality with severe developmental patterning defects (48). In contrast, hypomorphic Tg737 mutations (Tg737orpk mice) that result in stunted and morphologically abnormal cilia cause skeletal patterning defects, polycystic kidney disease (PKD), hydrocephalus, pancreas and liver anomalies, and severe growth retardation with mutants normally dying within 2 weeks of birth (3, 9, 24, 39, 47).
A unifying theme of the soft tissue pathologies in Tg737orpk mutants is altered fluid and ion transport properties. Data suggest that such alterations lead to excess cerebrospinal fluid (CSF) accumulation in the ventricles of the brain and cystic lesions in the kidney, and impaired fluid movement into the pancreatic duct and biliary tree in the liver. The mechanism by which dysfunction of cilia results in altered fluid transport remains largely unknown. Analysis of this question has been hindered by the embryonic lethality associated with complete cilia loss. Therefore hypomorphic mutations, such as the Tg737orpk mutant mouse, have become an essential resource to begin investigating the mechanisms connecting ciliary dysfunction to disease pathogenesis.

Previously, we described the hydrocephalus phenotype in the Tg737orpk mice. Hydrocephalus is a progressive pathological condition with a diverse etiology. The most common cause of hydrocephalus in humans and murine models is obstruction of the aqueduct that interconnects the brain ventricles. Less frequent causes are overproduction of CSF by choroid plexus and decreased reabsorption by arachnoid granulae. Interestingly, the etiology of hydrocephalus in Tg737orpk mice is not associated with duct stenosis or impaired CSF flow, but rather with abnormalities in CSF production. The pathology is then aggravated by the disorganized, slow motility of the cilia found on the ependymal cells lining the ventricles (3).

Homeostasis of the aqueous environment of the mammalian brain is maintained by choroid plexus epithelia (CPE) (38). Although, the composition of cerebrospinal fluid is similar to that of serum, it is not a clear ultrafiltrate, but the result of an active transport mediated by the CPE (8). The apical Na⁺/K⁺-ATPase is thought to be an important driving force for ion and fluid movement across CPE, which creates a continuous Na⁺
influx allowing other transporters to use this electrochemical gradient to transport ions and water. Na⁺-dependent HCO₃⁻ transporters play an important role in Na⁺ and HCO₃⁻ secretion into the CSF and maintenance of pHᵢ of the CPE. Currently three Na⁺-dependent HCO₃⁻ transporters have been described in the CPE: electroneutral NBCn1 and NCBE are located on the basolateral membrane and electrogenic NBCe2 on the apical side (7, 27).

In many transport epithelia ion and fluid secretion is regulated in part by cAMP (2, 11, 12, 44). Alterations in cAMP-dependent fluid movement has been implicated in the pathogenesis of several diseases such as polycystic kidney disease. The increased intracellular cAMP levels in cystic epithelium are associated with excess fluid secretion into the tubular lumen and increased epithelial cell proliferation. These are together thought to be a mechanism leading to cyst formation and expansion. Importantly, approaches to inhibit the increased cAMP levels with vasopressin V2 receptor (V2R) antagonists have been found to ameliorate cystic kidney disease pathology in several mouse models (42, 43).

Previously, we demonstrated that the defects in the cilia on CPE of Tg737orpk mutant mice are associated with markedly elevated levels of intracellular cAMP (3). However, it was not clear whether elevated cAMP levels had any relevance to the hydrocephalus pathology in the mutant animals. In the present study, we begin to address this issue by using ratiometric fluorescence imaging to examine and compare steady-state pHᵢ and Na⁺-dependent transport activities in the CPE of Tg737orpk and wild-type mice, and investigate the consequence of changing cAMP levels on these transport activities.
Our data suggest that cAMP-mediated signaling is a pathogenic mechanism leading to excess CSF production and the development of hydrocephalus.
Materials and Methods

Reagents

The dye 2',7'-bis (2-carboxyethyl)-5-(and-6) -carboxyfluorescein, acetoxymethyl ester (BCECF/AM), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt (DIDS) and nigericin were purchased from Molecular Probes, Inc. (Carlsbad, CA). Dibutyryl-cAMP and H-89 were purchased from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma (Saint Louis, MO).

Mice

Tg737orpk mice were generated as described previously (24). The lines were maintained as heterozygous crosses on an inbred FVB/N genetic background. Animals were treated and maintained in accordance with the IACUC regulations at the University of Alabama at Birmingham. Genotyping was performed as described previously (46).

Tissue preparation and measurement of pHᵢ

Day 5 and 6 wild-type and Tg737orpk littermates were sacrificed, choroid plexi were removed from the lateral ventricles and placed into a cooled dissection chamber filled with saline solution. Two tissue pieces, freshly isolated from similar regions of choroid plexi obtained from a mutant and wild-type animal were transferred to a thermo-regulated microscope chamber. The preparations were immobilized with glass micropipettes in a position where the epithelium of the two tissue pieces were facing each other (Figure 2). This allowed simultaneous imaging of the two preparations. The tissues were then loaded with BCECF by incubating them in a saline solution containing 10 µmol/L BCECF/AM.
for 20 minutes. Residual non-hydrolyzed dye was removed before the experiment by flowing saline solution at 2 ml/min for 5 min. During the experiment the bathing solution was exchanged at a rate of 2.5 ml/min. pHi was measured using a Nikon S Fluor 40x objective and assessed with dual excitation wavelength fluorescence system, which included a computer-controlled chopper assembly (530 nm emission during alternating 440 and 495 nm light excitation; Photon Technology International, West Sussex, UK) and a cooled SenSys charge-coupled camera (Photometrics, Tucson, AZ). Every experiment was calibrated using two pH points with the high-potassium/nigericin technique as described (41). For each experiment the 495/440 nm ratios were converted to pHi. All experiments were performed at 37 °C.

Buffering capacity and $HCO_3^-$ flux

To evaluate acid-base transporter activities, we determined the intrinsic buffering power in both wild-type and Tg737orpk choroid plexus tissues using the weak acid NH$_4^+$ (4, 6). These experiments were performed in the nominal absence of CO$_2$/HCO$_3^-$ and in the absence of external Na$^+$ to minimize the activity of pH$_i$ regulatory mechanisms. pH$_i$ was measured in response to stepwise changes in bath NH$_4$Cl concentration from 20, 10, 1 to 0 mM, and the intrinsic buffering power was calculated at the mean pH$_i$ between steps using the following equation:

$$\beta_i = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i$$

where $\beta_i$ is the intrinsic buffering capacity, $\Delta[\text{NH}_4^+]_i$ is the difference in intracellular ammonium concentration (calculated using $pK_a$ of 8.9 for NH$_4^+$) and $\Delta\text{pH}_i$ is the measured change in pH$_i$. 
As shown in Figure 1, the intrinsic buffering capacity displayed a linear decrease with higher pH\textsubscript{i} values. The intrinsic buffering power values at varying pH\textsubscript{i} were not significantly different in the wild-type and mutant CPE. We therefore pooled the data from both tissues and the intrinsic buffering power (in mmol/L) as a function of pH\textsubscript{i} was best fit with the equation:

\[ \beta_i = -107.54 \times \text{pH}_i + 768.03 \]

The initial net acid extrusion (\(J_{\text{net}}\)) was calculated using the following formula:

\[ J_{\text{net}} = \frac{d\text{pH}_i}{dt} \times (\beta_i + \beta_{\text{CO}_2}) \]

where \(d\text{pH}_i / dt\) is the initial rate of change in pH\textsubscript{i} (over 30 seconds) and \(\beta_{\text{CO}_2}\) is the buffering capacity conferred by bicarbonate, computed from the theoretical relationship:

\[ \beta_{\text{CO}_2} = 2.3 \times [\text{HCO}_3^-]_i \]

**Solutions**

Table 1 provides the composition of each solution used in our experiments. The pH of the solutions was adjusted to 7.4 at 37 °C. The osmolarity of all solutions was determined with a freezing point depression osmometer and was adjusted with mannitol.

**Statistical analysis**

Paired Student’s \(t\)-test was used for data analysis, where \(P < 0.05\) was considered significant. The data from the intrinsic buffering capacity experiments were analyzed by two-way repeated measures ANOVA.
Results

Technique of simultaneous imaging of paired tissue preparations

Analysis of physiological parameters, such as pH, can have a high degree of experimental variability as a result of the assay procedures. This can make it difficult to assess the physiological significance of changes that result from experimental or genetic manipulations. Thus, we utilized a simultaneous imaging approach (presented in Figure 2) to directly compare pHi and Na+-dependent transport activities in Tg737orpk mutant and wild-type choroid plexus epithelium. With this technique, the choroid plexi from two groups of animals (e.g. wild-type and Tg737orpk) are simultaneously loaded, incubated, and imaged. This eliminates variability conferred by experiment-to-experiment differences due to loading conditions and timing of solution changes, etc, and allows a more direct comparison of data between samples.

Steady-state pH\textsubscript{i} in choroid plexus epithelia from wild-type and Tg737orpk mice in the presence or absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}

Baseline pH\textsubscript{i} is determined by the balance between acid-loading mechanisms (acid loading transporters and passive fluxes of acid-base equivalents) and acid-extruding mechanisms (acid-extruding transporters). The activity and expression of many of the acid-base transporters are regulated by intracellular cAMP (15, 16, 37). Thus, we first investigated the pH\textsubscript{i} in choroid plexus epithelium from mutant and wild-type mice. As shown in Figure 3, the steady-state pH\textsubscript{i} was found to be higher in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-buffered solutions than in HEPES-buffered solutions, and under both conditions, pH\textsubscript{i} was
significantly lower in choroid plexus epithelium obtained from Tg737orpk mutants than from wild-type animals.

Sodium-hydrogen antiporter activity in choroid plexus epithelia obtained from wild-type and mutant animals

The lower pH$_i$ in choroid plexus epithelial cells from mutant animals relative to wild-types could be due to either stimulation of acid-loading or inhibition of acid-extruding mechanisms. Na$^+$-H$^+$ antiporter is a potent acid-extruder involved in regulating pH$_i$ and maintaining cell volume in many tissues (5, 14, 30). We therefore examined Na$^+$-H$^+$ antiporter activity in both mutant and wild-type CPE tissues (Figure 4). Tissues in the nominal absence of CO$_2$/HCO$_3^-$ were first acidified using the NH$_4^+$-prepulse technique, and pH$_i$ recovery was blocked by removing external Na$^+$. Na$^+$ was then readded and the initial rate of pH$_i$ recovery was used as an index of Na$^+$-H$^+$ antiporter activity. Neither the wild-type nor the mutant CPE tissues showed appreciable increase in pH$_i$ upon returning Na$^+$ to acid-loaded tissues. Together these data suggest that altered pH$_i$ is not due to defects of Na$^+$-H$^+$ antiporter activity.

Na$^+$-HCO$_3^-$ cotransport in choroid plexus epithelia from wild-type and Tg737orpk mice

Na$^+$-HCO$_3^-$ cotransporters also play an important role in pH$_i$ regulation (32). To date, three types of Na$^+$-dependent HCO$_3^-$ transporters have been described in the choroid plexus epithelia. The two basolateral transporters - NCBE and NBCn1 - are suggested to play a role in Na$^+$ and HCO$_3^-$ influx into the cells, while the apical NBCe2 may participate in the efflux of these ions to the cerebrospinal fluid, however the role of this
latest transporter remains to be determined (7). We therefore compared the Na\(^+\)-dependent HCO\(_3\)\(^-\) transporter activity in Tg737\(^{orpk}\) mutant and wild-type tissues. We used an acid-loading protocol similar to that described above. However, upon removing NH\(_4\)\(^+\) and Na\(^+\), we simultaneously switched to a solution buffered with 5% CO\(_2\)/22 mM HCO\(_3\)\(^-\) (Figure 5A). As shown in Figure 5B, the net acid extrusion (\(J_{\text{net}}\)) was significantly higher in the mutant CPE as compared to wild-type. These \(J_{\text{net}}\) values primarily represent HCO\(_3\)\(^-\)-dependent transport since there is very little Na\(^+\)-dependent pHi recovery in the nominal absence of CO\(_2\)/HCO\(_3\)\(^-\).

The effect of DIDS on Na\(^+\)-dependent HCO\(_3\)\(^-\) transport was also determined. We used choroid plexus tissue dissected from the same animal and pretreated one sample with 500 μmol/L DIDS for 15 minutes. The Na\(^+\)-dependent HCO\(_3\)\(^-\) transport activity in DIDS-pretreated tissues was 87.3% ± 19.2% as compared to control (n=6).

**Effect of db-cAMP and H-89 on Na\(^+\)-dependent HCO\(_3\)\(^-\) cotransport in choroid plexus epithelium**

An increased level of cAMP has been reported to activate Na\(^+\)-dependent HCO\(_3\)\(^-\) transport in pancreas, intestine and corneal epithelia (2, 40, 45). Also, our previous data have indicated increased levels of cAMP in Tg737\(^{orpk}\) CPE (3). Thus, we tested the possibility that an increase in intracellular cAMP could stimulate Na\(^+\)-dependent HCO\(_3\)\(^-\) transport activity in CPE tissue from wild type mice similar to that seen in Tg737\(^{orpk}\) mutants. For this analysis, pairs of CPE tissues were isolated from wild-type mice. To evaluate cAMP responses, one of the tissues was pretreated with 1 mM dibutyryl-cAMP for 20 minutes prior to the experiment and the Na\(^+\)-dependent HCO\(_3\)\(^-\) transport activity
was compared to the control CPE. As shown in Figure 6A and B, the Na$^+$-dependent HCO$_3^-$ transporter activity was significantly higher in tissues pretreated with db-cAMP.

These data indicate that cAMP may be a modulator that leads to the altered Na$^+$-dependent HCO$_3^-$ transport observed in the mutant CPE. To further assess this possibility, we evaluated Na$^+$-dependent HCO$_3^-$ transport activity in mutant CPE under conditions where cAMP signaling was inhibited. This was done using H-89, a potent protein kinase A (PKA) inhibitor. As performed with the wild-type CPE explants, a paired prep technique was utilized where one mutant sample was pretreated with H-89. In all samples analyzed, the preincubation with H-89 resulted in a significantly lower Na$^+$-dependent HCO$_3^-$ transport activity as compared to the non-treated mutant tissue (Figure 6 C and D).

Together, these data support a mechanism by which loss of normal cilia function leads to elevated intracellular cAMP levels that cause defects in the regulation of Na$^+$-dependent HCO$_3^-$ transport.
Discussion

In our previous work we demonstrated that Tg737orpk mice develop hydrocephalus that is initiated in the perinatal period and likely involves ion and fluid transport defects across the choroid plexus. This pathology was associated with a marked increase in intracellular cAMP (3). Another phenotype exhibited by the Tg737orpk mutants is the development of cystic renal lesions in the kidney (24). While it is not known whether cAMP is elevated in the kidney of Tg737orpk mutants, it is noteworthy that the cystic kidneys in human ARPKD and ADPKD patients and in many of the PKD mouse models caused by abnormal cilia function do have increased intracellular cAMP. Furthermore, inhibition of cAMP signaling using vasopressin receptor antagonists in mice with PKD greatly improves renal function and pathology and is currently being evaluated as a means of retarding cyst progression (42, 43). Together, these data suggested that cAMP may be central to the initiation of hydrocephalus and other pathological alterations in the kidney, liver, and pancreas of the Tg737orpk mutant mice.

cAMP is known to be an intracellular regulator of ion and water transport in many secretory/reabsorptive epithelia (e.g. pancreas, intestine, cornea, and kidney), and there are data to indicate a role for cAMP in ion and water transport across the CPE (2, 11, 12, 18, 45). In frog CPE, cAMP has been proposed to increase HCO₃⁻ secretion and ion transport into CSF; furthermore in mice there is an apically localized inward rectifying chloride channel that is activated by cAMP and cAMP agonists (8, 33, 34). Thus, in this work we evaluated whether altered cAMP levels observed in the CPE might contribute to changes in pHᵢ and ion transport activities that could explain an increase in CSF production and hydrocephalus in the Tg737orpk mutants.
To better compare acid-base transport mechanisms in mutant and wild-type CPE and their responses to cAMP, we utilized an imaging technique with paired tissue preparations where two tissues loaded with BCECF were assayed simultaneously, minimizing variance between experiments. First, steady-state pH<sub>i</sub> was measured in both mutant and wild-type tissues either in the absence or presence of CO<sub>2</sub>/HCO<sub>3</sub>−. CPE from Tg737<sup>orpk</sup> compared to wild-type mice had a lower pH<sub>i</sub> in both buffer conditions. We hypothesized that this was due to altered activity of either acid loading or extruding transporters. Current data indicate that CPE possess a Na<sup>+</sup>/H<sup>+</sup> antiporter (NHE1), Na<sup>+</sup>-dependent HCO<sub>3</sub>− transporters, and a Cl<sup>−</sup>/HCO<sub>3</sub>− exchanger (AE2) whose activity could contribute to the altered pH<sub>i</sub> regulation (8). Thus, a potential caveat that must be noted in this study is that we do not know whether there is a different profile of transporters expressed in mice at the ages used to evaluate CPE transport properties in the Tg737<sup>orpk</sup> mice.

To begin evaluating the cause of the difference in pH<sub>i</sub>, we first analyzed the activity of Na<sup>+</sup>/H<sup>+</sup> exchanger in mutant and wild-type tissues. However, based on our data, we could not detect a Na<sup>+</sup>-dependent pH<sub>i</sub> recovery following CPE acidification in the nominal absence of CO<sub>2</sub>/HCO<sub>3</sub>−. This would be expected if an active NHE were present on the plasma membrane. Thus, it is unlikely that this transport would contribute remarkably to the observed differences between pH<sub>i</sub> in CPE obtained from wild-type and mutant animals. Also, published data regarding the presence or activity of NHE in CPE are controversial. In two studies, a basolateral amiloride-sensitive NHE was suggested to participate in Na<sup>+</sup> uptake into CPE, assuring basolateral Na<sup>+</sup> supply in response to apical Na<sup>+</sup> flux into the CSF (10, 25). However, other groups found no evidence for the
expression of a Na⁺-H⁺ antiporter in choroid plexus suggesting that this antiporter may not be present or that another variant may exist (1, 28).

To further investigate the mechanism behind the pHᵢ differences, we compared Na⁺/HCO₃⁻ cotransporter activity in CPE from mutant and wild-type animals. There are three Na⁺/HCO₃⁻ cotransporters on CPE. Two are localized to the basolateral membrane (NBCn1 and NCBE) while the other is present on the apical membrane (NBCe2) (7). They all have important roles in regulating pHᵢ. In addition to pHᵢ regulation, the apically localized NBCe2 is thought to contribute directly to CSF production (28). In our studies, we found a marked increase in pHᵢ and calculated HCO₃⁻ flux was present in both mutant and wild-type samples following Na⁺ addition to acidified CPE tissues. In addition, the Na⁺-dependent HCO₃⁻ flux was significantly higher in the mutant versus wild-type samples. Based on our findings the activity of Na⁺/HCO₃⁻ cotransport of CPE showed little DIDS sensitivity at low intracellular pHᵢ.

We also investigated whether intracellular cAMP, which is markedly elevated in Tg737orpk mutant choroid plexus, was able to influence Na⁺-dependent HCO₃⁻ transport. cAMP is known to stimulate HCO₃⁻ flux in cornea, pancreas, and colon epithelial tissues. However, little is known about cAMP-mediated HCO₃⁻ transport in the mammalian choroid plexus. In our studies, we found that the addition of db-cAMP to wild-type CPE does result in significantly higher Na⁺-dependent HCO₃⁻ flux in acidified CPE tissues compared to untreated control samples. These data confirm that cAMP is able to regulate Na⁺-dependent HCO₃⁻ activity in the CPE and suggest that the elevated level of cAMP observed in the mutant CPE may stimulate Na⁺-dependent HCO₃⁻ transport. To further explore this possibility, we next treated mutant CPE explants with H-89, which blocks
cAMP mediated effects through inhibition of protein kinase A activity. The results from these experiments indicate that blocking PKA activity markedly reduced Na\(^+\)-dependent HCO\(_3\)- transport in mutant tissue. Taken together, these data raise the possibility that aberrant cAMP/PKA mediated signaling activity is a driving force in hydrocephalus of \(Tg737\text{orpk}\) mutants as recently suggested for cyst development in PKD, a phenotype also present in \(Tg737\text{orpk}\) animals.

The mechanism by which the impaired ciliary function on the CPE in the \(Tg737\text{orpk}\) mutant results in excess CSF is currently unknown. However, our data suggest at least two possibilities. In the first scenario, the cAMP mediated increased Na\(^+\)-dependent HCO\(_3\)- transport could be the driving force that leads to the excess CSF production. Increased Na\(^+\)-dependent HCO\(_3\)- transport activity in the mutants would cause a net increase in ion transport and subsequent fluid movement into the CSF. Indeed, data from frog CPE have already established a connection between cAMP and increase HCO\(_3\)- secretion into CSF that does lead to an increase in CSF production (33, 34). However, we note that the intracellular pH is low in our experimental conditions, well beyond the physiological range, consequently, the direction of the Na\(^+\)-dependent HCO\(_3\)- transport is inward in acidified CPE cells. In vivo, this is likely different, since at the estimated reversal potential of ~ -50 mV the transporter could be driven in either direction, depending on ion/Vm conditions (23, 29). The lower pH\(_i\) of the mutant CPE could be due to an increased acid loading by the apical Na\(^+\)/HCO\(_3\)- transporter: however, based on our data, we cannot identify the localization of the Na\(^+\)/HCO\(_3\)- transporter activated by cAMP. We also note here, that in our experimental conditions, the monitored region of interest of CP tissue is an intact epithelium, thus, epithelial responses
to the change of bathing solution are probably apical events. However, since our analysis is conducted on relatively small tissue samples with cut edges, we cannot exclude accessibility of the bathing solutions to the basolateral side of the CPE which could also contribute to the responses seen in our analyses.

In the second scenario we also propose a cAMP-driven effect on ion and water transport across the mutant CPE. In this case, the increased ion transport would be mediated by the apically localized inward rectifying chloride channel (ClC2 like channel). The ClC2-like channel transports both Cl⁻ and HCO₃⁻ into the CSF and is known to be stimulated by cAMP (8, 20, 21). Our previous studies have shown that Cl⁻ levels in the CSF are elevated in the mutants (3). Thus, cAMP induced activity of this channel could explain the changes in CSF chloride levels and lower pHᵢ observed in the mutants with the altered Na⁺-dependent HCO₃⁻ transport being a compensatory mechanisms responding to altered pHᵢ. The connection between cAMP and the activity of these transporters and channels and whether the altered activity results in increased CSF production in the Tg737orpk mutants is currently being evaluated.

As indicated above, increased cAMP levels are a pathogenic factor leading to the development of cystic kidney disease in ARPKD and ADPKD patients and animal models (42). In the renal cystic epithelia, adenylyl cyclase activity was elevated through the vasopressin V2 receptor (V2R). Furthermore, progression of the cystic pathology can be greatly retarded by the use of V2R antagonists (43). While V2R is not thought to be expressed in adult CPE, the mRNA was reported in the CPE of newborn rodents (19). Thus, it would be interesting to determine whether V2R expression is maintained in...
mutant CPE, and whether hydrocephalus pathology can be ameliorated through administration of V2R antagonists.

In summary, our data indicate that loss of normal function of the ciliogenic protein polaris in Tg737orpk mutant mice results in a lower steady state pHᵢ and higher Na⁺-dependent HCO₃⁻ transport activity in CPE. These changes are associated with elevated levels of intracellular cAMP in the mutant tissue. Indeed, addition of a cAMP analogue was able to increase Na⁺-dependent HCO₃⁻ transport in wild-type CPE, while H-89 an inhibitor of cAMP mediated PKA activity was able to reduce HCO₃⁻ flux in mutant tissue. We are currently evaluating whether the alteration in cAMP-mediated effects on Na⁺/HCO₃⁻ transport activity is associated with increased CSF production that could lead to development of hydrocephalus in these mice.
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Figure legends

**Figure 1** Graph showing the intrinsic buffering capacity of the choroid plexus epithelia from wild-type and *Tg737orpk* mice as a function of pH. Data points represent the average values from 3 pairs of preparations. Line represents linear fit to the pooled data from wild-type and mutant tissues.

**Figure 2** Freshly isolated *in vitro* choroid plexus preparations from wild-type and *Tg737orpk* mutant mice visualized simultaneously in one image field. (A) Bright-field image showing the wild-type and mutant choroid plexus tissues immobilized by micropipettes. Arrowheads indicate the epithelial cells at the edge of the choroid plexus. (B) Wide-field fluorescence image of the same tissues loaded with the intracellular pH sensitive dye BCECF. White rectangles represent the regions of interest corresponding to the epithelial cells. Scale bar denotes 40 µm.

**Figure 3** Measurement of intracellular pH in choroid plexus epithelia. (A) Representative traces demonstrating steady-state pH in choroid plexus epithelia (CPE) from simultaneously imaged wild-type and *Tg737orpk* mutant tissues in the absence or presence of CO₂/HCO₃⁻ in the bath. (B) Graphs showing pH values in CPE in the absence (circles) or presence (squares) of CO₂/HCO₃⁻ from wild-type and mutant animals. Filled symbols denote pH values from individual paired preparations, and open symbols show the average values ± SE (n=5; the values in the wild-type and mutant groups were different from each other under both conditions).
Figure 4  Dependence of pH$_i$ on extracellular Na$^+$ in choroid plexus epithelia (CPE) following an acid load in the absence of CO$_2$/HCO$_3^-$ . Representative traces of Na$^+$-dependent pH$_i$ recoveries following an intracellular acidification imposed by prior Na$^+$ removal for wild-type (solid line) and Tg737orpk (dashed line) choroid plexus tissues.

Figure 5  Dependence of pH$_i$ on extracellular Na$^+$ in choroid plexus epithelia (CPE) following acid load in the presence of CO$_2$/HCO$_3^-$ . (A) Representative traces of Na$^+$-dependent pH$_i$ recoveries following an intracellular acidification imposed by prior Na$^+$ removal for wild-type (solid line) and Tg737orpk (dashed line) choroid plexus tissues. The initial rate of pH change was measured over 30 seconds. (B) Net Na$^+$-dependent acid extrusion flux ($J_{net}$) after an acid load in wild-type and Tg737orpk choroid plexus tissues. Filled circles denote pH$_i$ values from individual paired preparations, and open circles show the average values ± SE (n=5; the values in the wild-type and mutant groups were different from each other).

Figure 6  Effect of intracellular cAMP concentration on Na$^+$-dependent pH$_i$ recovery after intracellular acidification in choroid plexus epithelia (CPE) in the presence of CO$_2$/HCO$_3^-$ . (A) Representative traces of Na$^+$-dependent pH$_i$ recoveries following an intracellular acidification imposed by prior Na$^+$ removal for control wild-type (solid line) and db-cAMP treated wild-type (dashed line) choroid plexus tissues. The initial rate of pH change was measured over 30 seconds. (B) Net HCO$_3^-$ flux after Na$^+$-addition in control and db-cAMP treated preparations (n=8; the values in the control and db-cAMP treated groups were statistically different from each other). (C) Representative traces of
Na⁺-dependent pHᵢ recoveries following an intracellular acidification imposed by prior Na⁺ removal for non-treated *Tg737orpk* mutant (solid line) and H-89 treated *Tg737orpk* mutant (dashed line) choroid plexus tissues. (D) Net HCO₃⁻ flux after acid load in non-treated and H-89 treated *Tg737orpk* mutant choroid plexus tissues. Filled circles denote pHᵢ values from individual paired preparations, and open circles show the average values ± SE (n=5; the treated and non-treated groups were statistically different from each other)

**Table 1** Composition of experimental solutions used in the study.
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<th>Materials (mmol/L)</th>
<th>145 NaCl</th>
<th>30 NH₄Cl</th>
<th>0 Na and HCO₃⁻</th>
<th>0 Na and HCO₃⁻</th>
<th>0 Na and 20 NH₄Cl</th>
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</tbody>
</table>
Figure 1

![Graph showing intrinsic buffer capacity vs. intracellular pH]
Figure 2
Figure 3

A

B
Figure 4

[Graph showing intracellular pH over time with different conditions indicated.]
Figure 5

A

B

Intracellular pH

Time (min)

Na\(^+\)

Na\(^+\) free

Na\(^+\)

NH\(^+\)

Tg737\(^{dupk}\)

Wild-type

0

2

4

6

J_{\text{net}} (\text{mmol L}^{-1} \text{ s}^{-1})

Wild-type Tg737\(^{dupk}\)

0.0

0.4

0.8

1.2
Figure 6

A

![Graph A showing intracellular pH over time with Na⁺, Na⁺ free, NH₄⁺, cAMP, and Control conditions.](image)

B

![Graph B showing J_net (mmol L⁻¹ s⁻¹) with Control and cAMP conditions.](image)

C

![Graph C showing intracellular pH over time with Na⁺, Na⁺ free, NH₄⁺, Control, and H-89 conditions.](image)

D

![Graph D showing J_net (mmol L⁻¹ s⁻¹) with Control and H-89 conditions.](image)