Basolateral Cl\(^{-}\) uptake mechanisms in *Xenopus laevis* lung epithelium

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Berger J, Hardt M, Clauss WG, Fronius M. Basolateral Cl\(^{-}\) uptake mechanisms in *Xenopus laevis* lung epithelium. *Am J Physiol Regul Integr Comp Physiol* 299: R92–R100, 2010. First published April 21, 2010; doi:10.1152/ajpregu.00749.2009.—A thin liquid layer covers the lungs of air-breathing vertebrates. Active ion transport processes via the pulmonary epithelial cells regulate the maintenance of this layer. This study focuses on basolateral Cl\(^{-}\) uptake mechanisms in native lungs of *Xenopus laevis* and the involvement of the Na\(^+/K^+\)/2 Cl\(^{-}\) cotransporter (NKCC) and HCO\(_3^/-\)/Cl\(^{-}\) anion exchanger (AE), in particular. Western blot analysis and immunofluorescence staining revealed the expression of the NKCC protein in the lung. Ussing chamber experiments demonstrated that the NKCC inhibitors (bumetanide and furosemide) were ineffective at decreasing the short-circuit current (ISC). The effect of DIDS was diminished by acetazolamide and reduced by increased external HCO\(_3^/-\) concentrations. Cl\(^{-}\) secretion induced by forskolin was decreased by DIDS, but this effect was abolished in the presence of forskolin and chlorzoxazone application. However, functional evidence for the NKCC was detected by generating a transepithelial Cl\(^{-}\) gradient. Further, we were interested in the involvement of the HCO\(_3^/-\)/Cl\(^{-}\) anion exchanger to transepithelial ion transport processes. Basolateral application of DIDS, an inhibitor of the AE, resulted in a significantly decreased short-circuit current (Isc). The effect of DIDS was diminished by acetazolamide and reduced by increased external HCO\(_3^/-\) concentrations. Cl\(^{-}\) secretion induced by forskolin was decreased by DIDS, but this effect was abolished in the presence of HCO\(_3^/-\). These experiments indicate that the AE at least partially contributes to Cl\(^{-}\) secretion. Taken together, our data show that in *Xenopus* lung epithelia, the AE, rather than the NKCC, is involved in basolateral Cl\(^{-}\) uptake, which contrasts with the common model for Cl\(^{-}\) secretion in pulmonary epithelia.

pulmonary ion transport; Cl\(^{-}\) secretion; Na\(^+/K^+\)/2 Cl\(^{-}\) cotransporter; HCO\(_3^/-\)/Cl\(^{-}\) anion exchanger

AIR-BREATHING VERTEBRATES developed lungs as gas-exchanging organs. Although breathing air gives almost infinite access to oxygen, it also bears some risks, represented by pollutants and pathogens, which are inhaled with each breath (24). Thus, the air-exchanging structures must fulfill two basic requirements: 1) they must be thin (41) for effective gas diffusion and 2) they must be strong to be an effective protective barrier (28). Interestingly, some features connected to these basic requirements for the function of the lungs are highly conserved within the vertebrates. On the one hand, the architecture of the gas-exchanging region, which is referred to as “three-ply design” (28), is realized in all air-breathing vertebrates. On the other hand, all air-breathing vertebrates possess a surfactant system as a part of the lung-lining fluid (LLF), covering the entire pulmonary epithelia (9).

It is obvious that the LLF is an important determinant for efficient lung function. Gas exchange and pulmonary immune defense depend on a defined composition, volume, and viscosity of the fluid layer covering the pulmonary tract. Maintenance and regulation of the LLF are achieved by transepithelial ion transport mechanisms (29). The net movement of ions across the epithelium generates osmotic gradients, and this represents the driving force for the osmotically driven water transport across the epithelium. Predominantly, the balance between Na\(^+\) absorption and Cl\(^{-}\) secretion determines the fluid content of the LLF (5, 10). The significance of pulmonary ion transport is underlined by human diseases as, for example, cystic fibrosis and pulmonary edema (10, 30). Both are associated with impaired pulmonary ion transport and altered composition of the LLF. Regarding the fatal consequences of an impaired pulmonary ion transport, it seems reasonable to assume that the evolutionary process of water-land transition must have been accompanied by the development of appropriate pulmonary ion transport mechanisms. Changes of the water content in the composition of the LLF would affect gas exchange, as well as immune defense, and both functions are crucial for survival.

At the moment, little is known about ion transport in non-mammalian lungs. Therefore, the present study facilitates the use of lung preparations of the South African clawed frog (*Xenopus laevis*) for pulmonary ion transport investigations. Two main reasons account for this aim: 1) as mentioned, comparatively little is known about pulmonary ion transport in non-mammalian air-breathing vertebrates and 2) in contrast to lungs of higher vertebrates, the simple sac-like anatomy of the anuran lung is suitable for electrophysiological recordings using Ussing chambers (4).

Although different studies already characterized the Na\(^+\) absorption and its regulation in *Xenopus* lung epithelium (2, 12, 13), little is known about Cl\(^{-}\) transport in this tissue. The importance of Cl\(^{-}\) secretion by pulmonary epithelial cells is obviously indicated by the pulmonary disease as observed in humans with cystic fibrosis. Mutations in the CFTR gene (cystic fibrosis transmembrane conductance regulator) lead to a defective Cl\(^{-}\) secretion that leads to an impaired mucociliary clearance, resulting in chronic infections and the subsequent formation of fibrosis. Secretion of Cl\(^{-}\) at the apical side of the tissues is suggested to be dependent on basolateral uptake of the anion involving cotransporter proteins, such as the Na\(^+/K^+\)/2 Cl\(^{-}\) cotransporter (NKCC) and/or the HCO\(_3^/-\)/Cl\(^{-}\) anion exchanger (26, 40). There is further evidence suggesting that the uptake of Cl\(^{-}\) at the basolateral side might determine the rate of Cl\(^{-}\) secretion (31), and this is crucial for the maintenance of the LLF and mucociliary clearance.

In a prior study the ability of the *Xenopus* lung epithelium for Cl\(^{-}\) secretion has been shown, including the expression and function of the CFTR in the *Xenopus* lung (39). Secretion of Cl\(^{-}\) via apical Cl\(^{-}\) channels must involve basolateral uptake mechanisms for Cl\(^{-}\) ions. Therefore, the present study focuses on the identification and characterization of Cl\(^{-}\) uptake mecha-...
anisms in this native lung tissue, especially regarding the role of basolateral NKCCs and HCO₃⁻/Cl⁻ anion exchangers.

**MATERIALS AND METHODS**

In general, for all experiments, freshly isolated lungs derived from adult female *X. laevis* were used (Kaehler, Germany or Xenopus-Express, France). Animals were kept in tap water and fed twice a week with commercial fish food. For our investigations, the animals were cooled down by placing them into ice water to make them manageable. Then, the animals were killed by decapitation, and the spinal cord was immediately pithed. Afterward, the lungs were dissected and either used for electrophysiological Ussing chamber measurements or for molecular investigations. All treatments of the animals were in agreement with the German law of animal care and were approved by the Regional Board Giessen.

Western blot analysis. Either frozen lungs or oocytes (positive control) from *X. laevis* (positive control) were homogenized in liquid nitrogen. The homogenates were resuspended in lysis buffer ([150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% NP40 (detergent), Na₃VO₄ (10 μM lysis buffer)] and Complete (protease inhibitor, 40 μl/ml lysis buffer; Roche Diagnostics, Mannheim, Germany) and dispersed via a syringe. Samples were centrifuged for 15 min at 4°C (13,000 rpm) to obtain a protein-containing supernatant, which was then denatured for 15 min at 95°C. Subsequently, 20 μl of each homogenate was separated on a 10% SDS polyacrylamide-gel membrane. The homogenates were resuspended in lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% NP40 (detergent), Na₃VO₄ (10 μM lysis buffer)) and Complete (protease inhibitor, 40 μl/ml lysis buffer; Roche Diagnostics, Mannheim, Germany) and dispersed via a syringe. Samples were centrifuged for 15 min at 4°C (13,000 rpm) to obtain a protein-containing supernatant, which was then denatured for 15 min at 95°C. Subsequently, 20 μl of each homogenate was separated on a 10% SDS polyacrylamide-gel membrane. The membrane was incubated with 5% nonfat dry milk PBST (phosphate saline buffer + 0.1% Tween20) solution for 1 h to prevent unspecific binding. Next, the membrane was incubated overnight at 4°C with the first antibody [1:1,000; T4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa] was incubated for 1 h at room temperature followed by three final washing steps with PBS (0.1 M), each for 5 min. Sections were investigated with a fluorescence microscope (Olympus AX70; Olympus, Tokyo, Japan) and analyzed with the analySIS software (Olympus).

Ussing chamber experiments. Following dissection, the lungs were incised from their proximal to their distal end, mounted plane between two plastic rings (one equipped with needles, the other one with compatible holes), and transferred into a modified Ussing chamber. The aperture of the chamber was 0.5 cm², defining the measuring area of the epithelium.

The Ussing chamber was connected with the voltage-clamp amplifier via custom-made electrodes. The electrodes were made of modified 200-μl pipette tips used as holders. The tips were filled with 1 M KCl agar (3%), which was covered by 1 M KCl solution. Ag/AgCl wires were inserted in this KCl solution and were connected with the voltage-clamp amplifier (custom-made, University of Hohenheim, Hohenheim, Germany). Only electrode pairs with a spontaneous potential less than 1.0 mV were accepted for use in the experiments.

Apical and basolateral compartments were perfused with NRS containing (in mM): 100 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, 10 glucose (pH 7.4). For some experiments, Cl⁻-free solution was used for perfusion of the apical compartment in which the Cl⁻ salts were replaced with equimolar concentrations of the appropriate gluconate salts. HCO₃⁻ solution contained (in mM) 75 NaCl, 25 NaHCO₃, 3 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, and 10 glucose (pH 7.4). After mounting and starting perfusion of the tissue, an initial transepithelial voltage (Vt, lumen negative) was measured. Following a short equilibration period of about 5 min, the VT was clamped to 0 V, and the required short-circuit current (ISC) was recorded continuously on a PC, as well as on an analog strip chart recorder. After a further equilibration period of ~120 to 240 min and achievement of a constant ISC value, experiments were started by application of drugs. Experiments were performed at room temperature.

Chemicals. The loop diuretics furosemide and bumetanide (both obtained from Sigma) were used as blockers of the NKCC. The secretagogues chlorozoxazone (Sigma) and forskolin (Tocris, Germany) were utilized to stimulate apical Cl⁻ secretion. As Cl⁻ channel blocker, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPBB, Tocris, Germany) was used. To investigate the involvement of the HCO₃⁻/Cl⁻ anion exchanger in Xenopus lung epithelium, DIDS (Sigma) was applied. Acetazolamide (Sigma) was used to inhibit endogenous hydrogen carbonate production by cellular carbonic anhydrase. Bumetanide, furosemide, forskolin, chlorozoxazone NPB, and DIDS were dissolved in DMSO (Fluka) and added from stock solutions. DMSO concentrations in the Ringer solution were generally ≤0.1%. Nevertheless, for each set of experiments, appropriate control experiments were performed using corresponding DMSO concentration, as used in the experiments with the drugs.

Data analysis and statistical evaluation. Data are presented as means ± SE; n marks the number of conducted experiments. ISC values were taken before and after the application of a drug to evaluate its effect on ion transport processes. In these cases, Student’s paired t-test was used to test whether means were significantly different. For comparison of effects induced by the application of a drug, differences were calculated from ISC values before and after drug application. Comparing drug-induced differences from independent experiments, the Mann-Whitney U-test was used. In all cases, a P value of at least ≤0.05 was considered as significantly different and marked (*P < 0.05; **P < 0.01; ***P < 0.001).

RESULTS

Effect of loop diuretics on transepithelial ion current. Considering that Xenopus lung epithelium is able to secrete Cl⁻ under basal conditions (39), we were interested in whether Cl⁻ uptake into the epithelial cells involves basolateral NKCCs. Blocking Cl⁻ uptake might result in a reduced Cl⁻ secretion, and this would induce a reduction of ISC. Ussing chamber
experiments were performed, elaborating the effect of the loop diuretics bumetanide and furosemide, common inhibitors of the NKCC (18). Following equilibration of the $I_{SC}$, either bumetanide (200 μM) or furosemide (200 μM) was added to the basolateral aspect of the tissues. However, under these basal conditions neither bumetanide ($n = 27$) nor furosemide ($n = 5$) had any effect on $I_{SC}$ (Fig. 1), negating the presence of NKCC, or its participation in $\text{Cl}^-$ uptake and secretion in *Xenopus* lung epithelium. Additional experiments were performed applying bumetanide in HCO$_3^-$ (25 mM) containing solution. Under these conditions, we also did not observe an effect by the loop diuretic (Fig. 1, D and E).

Next, NKCC expression in *Xenopus* lung epithelium was checked. Therefore, Western blot experiments from *Xenopus* lung homogenates of three different donor frogs were performed. In all three samples, the antibody detected a band with a molecular mass of $\sim 130$ kDa (Fig. 2A). This corresponds to the predicted size of the NKCC protein (18, 27, 37). Additionally, immunofluorescence stainings of *Xenopus* lung slices were performed to localize NKCC in the tissue. Particularly, the pneumocytes (pn), characterized by cell bodies between capillaries (c) and large, thin cell extensions covering the capillaries from the luminal side, exhibited areas of considerable staining (Fig. 2B). In control tissues, under omission of the first antibody, no fluorescence was detected (Fig. 2B, inset). These results indicate that the NKCC protein is expressed in *Xenopus* lung epithelium but not operating under basal conditions.

**Effect of loop diuretics under stimulated $\text{Cl}^-$-secreting conditions**. One of the most important triggers for the activation of the NKCC is represented by a decrease of the intracellular $\text{Cl}^-$ concentration [Cl$_i$] (20). This is usually achieved by activating apical $\text{Cl}^-$ channels and bases on the assumption that $\text{Cl}^-$ secretion requires basolateral $\text{Cl}^-$ uptake, and this would activate NKCC (20). In the present study, different attempts were used to increase $\text{Cl}^-$ secretion to prove the functional contribution of the NKCC. Chlorzoxazone was used to induce $\text{Cl}^-$ secretion, since it has been described as a secretagogue (6, 38). The application of chlorzoxazone (500 μM, basolateral) led to a significant increase of the $I_{SC}$, indicating an increased $\text{Cl}^-$ secretion (Fig. 3A). Further experiments were performed, in which the tissues were incubated with bumetanide prior to the application of chlorzoxazone (Fig. 3B). This maneuver is likely to prevent $\text{Cl}^-$ uptake via the NKCC (if activated) and should subsequently reduce $\text{Cl}^-$ secretion and thus the chlorzoxazone-induced current.

![Image](http://ajpregu.physiology.org/)

**Fig. 1.** Effect of loop diuretics on short-circuit current ($I_{SC}$) in *Xenopus laevis* lung epithelium. **A**: original current trace of a recording, elaborating the effect of bumetanide (bumet.; 200 μM basolateral) on $I_{SC}$ under basal conditions. **B**: statistical evaluation of data shown in A. Under these conditions, no changes in $I_{SC}$ were observed (ns: not significant; paired Student’s $t$-test). **C**: results from corresponding experiments using furosemide (furo.) instead of bumetanide. Means ($\pm$ SE) represent $I_{SC}$ values before (control) and after furosemide application (ns: not significant; paired Student’s $t$-test). **D**: current trace representing the effect of bumetanide on $I_{SC}$ under HCO$_3^-$ perfusion. Bumetanide did not affect $I_{SC}$ under these conditions. **E**: statistical evaluation of experiments as shown in D. Bumetanide was without effect on $I_{SC}$ (ns, not significant; paired Student’s $t$-test).
Interestingly, comparing the chlorzoxazone-induced current (I_{CHLOR}) under control conditions with I_{CHLOR}, estimated in the presence of bumetanide, no significant changes were detected (Fig. 3C). Identical experiments were performed with the secretagogue forskolin (10 \mu M, both sides of the tissue) to activate Cl^{-} secretion (1, 33). In these experiments, the forskolin-induced (IFOR) current was also estimated in the presence and absence of bumetanide, and again, no changes were observed (Fig. 3D). To estimate whether the secretagogues activate Cl^{-} secretion, experiments with the Cl^{-} channel blocker NPPB were performed. Therefore, the effect of NPPB (100 \mu M, apical side) was determined under control conditions, as well as after chlorzoxazone application (Fig. 3E). The NPPB-sensitive current was significantly more pronounced in chlorzoxazone-stimulated tissues, indicating the activation of Cl^{-} secretion by the secretagogue. The ability of forskolin to activate a Cl^{-}-secreting component in Xenopus lung epithelia has been demonstrated earlier (3). However, taken together, there is no evidence concerning the participation of NKCC in Cl^{-} secretion under pharmacologically stimulated (Cl^{-} secreting) conditions.

A more radical attempt to prove the function of the NKCC in Xenopus lung epithelium was achieved by apical perfusion of Cl^{-}-free solution (the Cl^{-} anion was replaced by gluconate). This procedure establishes a massive Cl^{-} gradient across the epithelium and induces increased Cl^{-} secretion (42). After reaching a stable ISC value under apical Cl^{-}-free conditions, the subsequent basolateral application of bumetanide (200 \mu M) significantly decreased ISC, indicating the activation of NKCC under these gradient conditions (Fig. 4, A and B). Further, the inhibition obtained with identical concentrations of furosemide (200 \mu M) also significantly decreased the ISC, although not to that extent as observed with bumetanide (Fig. 4B). This corresponds to the known affinity profile of the diuretics, where bumetanide is more potent than furosemide (18).

So far, the results obtained demonstrate the presence of the NKCC in Xenopus pulmonary epithelium, although that the
Evidence for the participation of HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} anion exchangers in Cl\textsuperscript{-} secretion. Since Cl\textsuperscript{-} secretion in Xenopus lung epithelia has been reported under basal conditions (39), alternative ways for the uptake of Cl\textsuperscript{-} must exist. A potential candidate is the HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} anion exchanger (AE), as identified in different mammalian airway epithelia (15, 26, 40). To evaluate the function of this, exchanger experiments with DIDS, a common blocker of the HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} anion exchanger (15), were performed (Fig. 5A). Basolateral application of DIDS (500 μM) decreased $I_{SC}$ by $-8 \pm 1\%$ (Fig. 5B, $P < 0.001$, $n = 12$), although the decrease was transient and usually compensated within 10–15 min. Since function of the AE might be influenced by the external HCO\textsubscript{3}\textsuperscript{-} concentration, identical experiments were performed with HCO\textsubscript{3}\textsuperscript{-}-containing solution (Fig. 5C). In these experiments, DIDS significantly decreased $I_{SC}$ by about $4.5 \pm 1\%$ (Fig. 5D, $P < 0.01$; $n = 8$). However, the initial decrease of the $I_{SC}$ as observed with DIDS (with and without HCO\textsubscript{3}\textsuperscript{-}) indicates the presence and function of the HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} anion exchanger.

To verify this assumption, experiments with acetazolamide, an inhibitor of the carbonic anhydrase (32), were performed. Perfusion of the tissues with 1 mM acetazolamide completely diminished the DIDS-induced effect. This was the case in the absence of HCO\textsubscript{3}\textsuperscript{-} (Fig. 6, A and B), as well as in the presence of 25 mM HCO\textsubscript{3}\textsuperscript{-} (Fig. 6, C and D).

To investigate a potential role of the anion exchanger under pharmacologically stimulated Cl\textsuperscript{-} secretion, again, we used forskolin (3). For this purpose, lung preparations were exposed to DIDS, and then forskolin (10 μM, both sides of the tissue) was added. After the transient DIDS effect was terminated, forskolin still induced a significant increase in $I_{SC}$. Comparing the forskolin-induced current ($I_{FOR}$) from control experiments (without DIDS) with $I_{FOR}$ during DIDS perfusion under NRS conditions, a significant reduction with DIDS was obtained (Fig. 7, A and B). Performing experiments with HCO\textsubscript{3}\textsuperscript{-}-containing solution, no DIDS effect was detected on the forskolin-induced current (Fig. 7, C and D). These observations confirm the ability of external HCO\textsubscript{3}\textsuperscript{-} to influence AE activity, as already indicated in Fig. 5, C and D.

Taken together, the experiments with DIDS indicate that the HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} anion exchanger is involved in Cl\textsuperscript{-} uptake under basal physiological conditions, as well as partially, under pharmacologically stimulated Cl\textsuperscript{-}-secreting conditions. Further, the HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} exchanger rather than the NKCC seems to participate in Cl\textsuperscript{-} uptake under physiological conditions.

**DISCUSSION**

Although lungs of tetrapods developed class- and order-specific anatomical adjustments during evolution, the thin fluid layer (lung lining fluid, LLF) covering the luminal surface of the pulmonary epithelia is highly conserved in air-breathing vertebrates (8). There is considerable evidence that the function of this layer is related to immune defense and gas exchange. This consideration derives from the fact that impaired
demonstrated the ability of the anions in native lung preparations of \( /H_11002 \) in the apical membrane of epithelial cells, as well as Cl absorption is crucial for the maintenance of the LLF properties.

A particular (15).

Fig. 6. Evidence for the involvement of the \( \text{HCO}_3^-/\text{Cl}^- \) anion exchanger in Cl uptake. A: acetazolamide, an inhibitor of the cellular carbonic anhydrase, was used to deplete cellular \( \text{HCO}_3^- \) delivery to reduce the activity of the \( \text{HCO}_3^-/\text{Cl}^- \) anion exchanger. Preincubation of the preparations with acetazolamide (1 mM, both sides) was found to abolish the effect of DIDS. B: bar graph illustrating the means (± SE) obtained without and after DIDS in the presence of acetazolamide (acetaz.). Application of DIDS was without any effect on \( I_{sc} \) (paired Student’s \( t \)-test). C: original current trace representing experiments as performed in A using \( \text{HCO}_3^- \)-containing solution. Under these conditions, acetazolamide still abolished the DIDS effect. D: statistical evaluation of experiments as shown in C. The application of DIDS resulted in no effect on \( I_{sc} \). Control values (before DIDS) were not different from values after DIDS application (paired Student’s \( t \)-test).

Volume and viscosity of the LLF is related to severe human diseases (10, 30). Cystic fibrosis, for example, is characterized by severe pulmonary disease caused by disturbances in pulmonary Cl transport due to a mutated CFTR Cl channel (5). Cl secretion by pulmonary epithelial cells is suggested to be the counterbalance of Na reabsorption, and it is further suggested that a defined balance between secretion and reabsorption is crucial for the maintenance of the LLF properties (29). The mechanism of Cl secretion involves Cl channels in the apical membrane of epithelial cells, as well as Cl uptake mechanism, which are yet less understood (16).

The present study focused on basolateral Cl uptake mechanisms in native lung preparations of \( X. laevis \). Prior studies demonstrated the ability of the \( Xenopus \) lung epithelium to secrete Cl (39). Commonly, for Cl-secretating epithelia, it is described that the \( \text{Na}^+/\text{K}^+/2\text{Cl}^- \) cotransporter (NKCC) facilitates Cl uptake and that the function of this cotransporter is crucial for Cl secretion via ion channels (16). This principle has been shown in a variety of secretory epithelia, including the shark rectal gland (27, 34), rat acinar salivary glands (35), human intestinal epithelial cells (7), and tracheal epithelium from dog and ferret (14, 20, 21).

Because of the widespread involvement of NKCC in Cl uptake in lung/airway epithelia of multiple mammalian species, expression of NKCC protein in the \( Xenopus \) lung was investigated. Western blot analysis and immunohistological staining clearly support the existence of the cotransporter in \( Xenopus \) lung epithelia. Partially, this is consistent with data from mice (36), demonstrating cotransporter expression “throughout” the lung, with highest expression levels in the proximal airways. Although expression of the NKCC protein in \( Xenopus \) lung was detected, it was difficult to observe functional contribution of the NKCC. The loop diuretics bumetanide and furosemide, common inhibitors of NKCC (18), were used for its functional detection. Neither under basal conditions nor with secretagogues (chlorzoxazone and forskolin) did we find evidence for the functional involvement of the NKCC in Cl secretion. On the one hand, this finding contrasts with studies describing that chlorzoxazone (1, 38, 39) and/or forskolin (1, 33) activates apical Cl secretion and that this effect was sensitive to bumetanide/furosemide, which is a hallmark for the participation of the NKCC (18). On the other hand, similar findings were reported from mouse trachea (16).

In that study, Grubb and colleagues demonstrated that loop diuretics neither affected Cl secretion under basal conditions, nor during forskolin-induced Cl secretion. A minor role for the NKCC in pulmonary ion transport can also be deduced from reports that NKCC knockout animals develop normal lungs and do not exhibit obvious abnormalities concerning pulmonary ion transport properties and Cl secretion, in particular (15).

Fig. 7. Involvement of the \( \text{HCO}_3^-/\text{Cl}^- \) anion exchanger in stimulated Cl secretion. A: experiment illustrating the effect of DIDS (500 \( \mu \)M, basolateral) on the forskolin-induced current increase. B: statistical evaluation comparing the forskolin-induced current \( (I_{FOR}) \) in the absence (control) and the presence of DIDS. \( I_{FOR} \) was decreased by DIDS preincubation (*) \( P < 0.05; \) Mann-Whitney \( U \)-test). C: current trace of experiments as conducted in A in the presence of external \( \text{HCO}_3^- \). Under \( \text{HCO}_3^- \) perfusion, DIDS application led to transient current decrease followed by an \( I_{sc} \) increase induced by additional forskolin application. D: statistics of experiments as conducted in C. Note that in the presence of increased external \( \text{HCO}_3^- \) concentrations, DIDS did not further affect \( I_{FOR} \) (Mann-Whitney \( U \)-test).

\[ \text{HCO}_3^- \text{uptake in XENOPUS LUNG EPITHELIUM} \]
Interestingly, the present study observed a loop diuretic-sensitive current by establishing a Cl⁻ gradient as achieved using apical Cl⁻-free solution. This finding confirms: 1) that NKCC is expressed in Xenopus lung epithelium, as indicated from the Western blot and immunostaining experiments, 2) that the cotransporter is basically able to operate, and importantly 3) that the loop diuretics are generally able to interfere with the Xenopus NKCC ortholog. Since function of the NKCC in Xenopus lung epithelium was only observed under Cl⁻ gradient conditions, which are not supposed to be relevant under normal physiological conditions, the role of the NKCC in Xenopus lung epithelium, in general, and its contribution to Cl⁻ secretion, in particular, remains uncertain. An explanation for the activation of the NKCC under apical Cl⁻-free conditions might be reasoned by the massive loss of cellular Cl⁻ due to the Cl⁻-free solution. It was shown that replacement of apical Cl⁻ with gluconate increased NKCC activity in primary canine tracheal and bronchial epithelial cells via cell shrinkage (19). Further, it has been demonstrated that NKCC activity is phosphorylation dependent and that the phosphorylation status is related to intracellular Cl⁻ concentrations (18). Thus, it could be hypothesized that reduced intracellular Cl⁻ concentrations correlate with NKCC phosphorylation and thus its activity. In Xenopus lung epithelium, the reduction of intracellular Cl⁻ by activating Cl⁻ secretion due to chlorzoxazone and forskolin preincubation was probably not strong enough to activate NKCC. From this point of view, it might be suitable to hypothesize that in Xenopus lung epithelium NKCC might rather be involved in cellular volume regulation, as probably obtained by replacing Cl⁻ with gluconate, than in basolateral Cl⁻ uptake for facilitating Cl⁻ secretion via apical channels. Regarding the inactivity of NKCC under physiological conditions, the question for alternative basolateral Cl⁻ uptake mechanisms arose. An appropriate candidate could be the HCO₃⁻/Cl⁻ exchanger. AE activity was observed in various airway epithelia (11), as well as in alveolar type II pneumocytes (23). Further significance concerning the importance of HCO₃⁻/Cl⁻ exchanger in pulmonary Cl⁻ transport is provided by studies using NKCC knockout mice (15). That study concluded that normal lung development in these animals might be due to the function of basolateral HCO₃⁻/Cl⁻ exchangers bypassing the loss of NKCC function.

In the present study, we got hints that the HCO₃⁻/Cl⁻ anion exchanger is involved in basolateral Cl⁻ uptake. In experiments using basolateral DIDS, an inhibitor of the HCO₃⁻/Cl⁻ exchanger (15), a significant decrease in Iₛₐₗ was observed, which corresponds to a decreased apical Cl⁻ secretion. This result implies the activity of a basolateral HCO₃⁻/Cl⁻ exchanger in Xenopus lung epithelium under basal conditions. This assumption was further supported by experiments using acetazolamide perfusion prior to DIDS application. Acetazolamide is a known inhibitor of carbonic anhydrase (15). Carbonic anhydrase is responsible for the production of HCO₃⁻, the intracellular substrate for the HCO₃⁻/Cl⁻ exchanger. Inhibition of carbonic anhydrase eliminates the delivery of HCO₃⁻ and reduces HCO₃⁻/Cl⁻ exchanger activity. This indication has been confirmed in the present study. Preincubation with acetazolamide diminished the DIDS effect, as observed under basal conditions.

To further confirm the role of the HCO₃⁻/Cl⁻ exchanger in stimulated Cl⁻ secretion, the secretagogue forskolin was used (1). Forskolin led to a robust increase of the Iₛₐₗ, at least partially representing an induced Cl⁻-secreting component, since it was shown that under Cl⁻-free conditions, the effect of forskolin was decreased compared with control condition (NRS) (3). In our experiments, incubation of the epithelia with DIDS prior to the application of forskolin was found to significantly decrease the forskolin effect when using NRS solution. This suggests that the HCO₃⁻/Cl⁻-exchanger is involved in Cl⁻ uptake under these conditions. The observation that forskolin was still able to increase short-circuit current in the presence of DIDS suggests that the forskolin-induced, Cl⁻-secreting component that was blocked with DIDS can be compensated by unknown Cl⁻ uptake mechanisms and confirms that forskolin, besides its ability to induce Cl⁻ secretion, also has the capability to increase Na⁺ absorption in Xenopus lung epithelium, the latter still present after AE inhibition by DIDS application.

Another possibility to verify the activity of the AE is represented by changing the availability of the substrate transported by the exchanger. Depletion of intracellular HCO₃⁻ (application of acetazolamide), as well as changes of extracellular HCO₃⁻ concentrations (25 mM), is suggested to affect the activity of AE. With both approaches, we were able to detect a reduction of the DIDS-sensitive component as a measure for AE activity. These observations clearly underline the function of the AE in Xenopus pulmonary epithelium, independent of whether the experimental solution contained HCO₃⁻ or not. The omission of the DIDS sensitivity of Iₙₒᵣₓ under HCO₃⁻ perfusion confirms the involvement of the HCO₃⁻/Cl⁻ exchanger, since we have shown that AE activity can be reduced by external HCO₃⁻ (Fig. 5, C and D). The fact that about 18% of the basal Iₛₐₗ could be blocked by apical Cl⁻ channel blockers (39) and that only 8% of the Iₛₐₗ are DIDS sensitive (Fig. 5B) further implies the existence of additional, yet unknown, Cl⁻ uptake mechanisms.

In summary, the present study identified the function of two different proteins in the lung epithelium of X. laevis involved in pulmonary ion transport processes. While the NKCC was found to be active solely under Cl⁻ gradient conditions, HCO₃⁻/Cl⁻ anion exchanger activity has been detected under basal conditions, as well as under forskolin-induced stimulation. These results demonstrate that the anion exchanger rather than the NKCC facilitates Cl⁻ uptake in pulmonary epithelial cells of Xenopus. From an evolutionary perspective, pulmonary ion transport properties in Xenopus lung might reflect the basic requirements for LLF regulation as an adaptation to air breathing. From this perspective, the present study might outline the importance of the anion exchanger, since, usually, it is suggested that the NKCC is the major player mediating pulmonary Cl⁻ transport. Nevertheless, the ability of the Xenopus lung epithelium to reabsorb Na⁺ and to secrete Cl⁻ corresponds well to the situation found in mammalian pulmonary epithelia. This indicates that the principle mechanisms, as well as the proteins involved in the maintenance of the LLF, are highly conserved in air-breathing vertebrates.

Perspectives and Significance

Studies concerning ion transport processes in pulmonary epithelia are mainly performed using isolated and/or cultured pulmonary epithelial cells. It is known that ion transport
processes of these epithelial cells can be altered by missing cell-cell interactions, by hormonal treatment, and especially by culture conditions (22, 25). Because of its relative simple anatomy, usage of native Xenopus lung preparations circumvents these disadvantages, especially regarding functional ion transport investigations by the Ussing chamber technique. The morphology of the air-blood barrier of X. laevis exhibits remarkable similarities compared with that in mammals, indicating a highly conserved architecture of the air-exchanging structures. We hypothesize that similar to the conserved anatomy of the air-blood barrier, the breathing of air is also related to the development of effective pulmonary ion transport processes in vertebrate evolution. From this point of view, investigations of pulmonary ion transport processes using amphibian lungs might, therefore, be beneficial and a useful tool for understanding pulmonary ion transport processes in higher vertebrates or even in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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