J. Membrane Biol. 192, 101–110 (2003)
DOI: 10.1007/s00232-002-1067-8

The Journal of
Membrane
Biology

Effects of Purinergic Stimulation, CFTR and Osmotic Stress on Amiloride-sensitive Na^+ Transport in Epithelia and *Xenopus* Oocytes

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Received: 26 July 2002/Revised: 18 November 2002

Abstract. Both stimulation of purinergic receptors by ATP and activation of the cystic fibrosis transmembrane conductance regulator (CFTR) inhibit amiloride-sensitive Na^+ transport and activate Cl^- secretion. These changes in ion transport may well affect cell volume. We therefore examined whether cell shrinkage or cell swelling do affect amiloride-sensitive Na^+ transport in epithelial tissues or *Xenopus* oocytes and whether osmotic stress interferes with regulation of Na^+ transport by ATP or CFTR. Stimulation of purinergic receptors by ATP/UTP or activation of CFTR by IBMX and forskolin inhibited amiloride-sensitive transport in mouse trachea and colon, respectively, by a mechanism that was Cl^- dependent. When exposed to a hypertonic but not hypotonic bath solution, amiloride-sensitive Na^+ transport was inhibited in mouse trachea and colon, independent of the extracellular Cl^- concentration. Both inhibition of Na^+ transport by hypertonic bath solution and ATP were additive. When coexpressed in *Xenopus* oocytes, activation of CFTR by IBMX and forskolin inhibited the epithelial Na^+ channel (ENaC) in a Cl^-dependent fashion. However, both hypertonic and hypotonic bath solutions showed only minor effects on amiloride-sensitive conductance, independent of the bath Cl^- concentration. Moreover, CFTR-induced inhibition of ENaC could be detected in oocytes even after exposure to hypertonic or hypotonic bath solutions. We conclude that amiloride-sensitive Na^+ absorption in mouse airways and colon is inhibited by cell shrinkage by a mechanism that does not interfere with purinergic and CFTR-mediated inhibition of ENaC.

Key words: CFTR — EnaC — X*enopus* oocytes — Mouse trachea — Cystic fibrosis — Cell swelling — Osmotic stress — UTP — ATP — Purinergic receptors — Epithelial transport

Introduction

Epithelial Na^+ channels (ENaC) are essential for absorption of Na^+ in epithelial tissues, where they colocalize with the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated Cl^- channel and a regulator of other channels [42]. Inhibition of amiloride-sensitive epithelial Na^+ channels by CFTR seems to be of highest physiological and pathophysiological relevance in cystic fibrosis [24, 26]. Due to the CFTR defect in cystic fibrosis and thus a lack of inhibition of ENaC, amiloride-sensitive Na^+ transport is enhanced in both airways and colon of CF patients [3, 5, 29, 31]. Apart from the regulation by CFTR, epithelial Na^+ transport is also inhibited by extracellular nucleotides such as ATP or UTP by a yet unknown mechanism. Purinergic inhibition of ENaC was detected in native and cultured epithelial cells from airways and collecting duct [9, 16, 27, 28, 33]. In the airways, stimulation of luminal purinergic receptors activates a transient Ca^{2+}-dependent Cl^- conductance of unknown molecular identity. In parallel to the activation of a Cl^- conductance, ATP inhibits amiloride-sensitive Na^+ absorption, a phenomenon that takes also place in CF airways [9, 33]. Inhibition of Na^+ absorption during stimulation of purinergic P2Y receptors is augmented by the parallel activation of Ca^{2+}-dependent Cl^- channels. However, Na^+ ab-

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc}, V_{te}/R_{te}, equivalent short circuit current; V_{te}, transepithelial voltage; $I_{sc\text{-}UTP}$, UTP induced I_{sc}; $I_{sc\text{-}Amil}$, amiloride-sensitive I_{sc}; PMA, 12-myristate 13-acetate Phorbolester; UTP, uridine 5′-triphosphate, ATP, adenine 5′-triphosphate.

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sorption is inhibited independently by an increase in intracellular Ca^{2+} or activation of protein kinase C [25, 33]. A similar Cl^--dependent inhibition of ENaC was found during activation of the CFTR Cl^--conconductance [6, 22].

Amiloride-sensitive Na^+ absorption and ENaC, respectively, were also shown to be affected by cell volume changes under hypotonic or hypertonic stress. However, studies directed at examining the mechanosensitivity of ENaC have produced conflicting results. When ENaC was expressed in *Xenopus* oocytes, the effects of osmotic stress on amiloride-sensitive Na^+ currents were rather variable [2, 17]. Similar contradictory results have been obtained in experiments on native Na^+ channels in various types of epithelia including cortical collecting duct, frog skin, hepatocytes, fetal distal lung epithelium and A6 cells [7, 34, 37, 38, 45]. Thus, epithelial Na^+ absorption and ENaC are either inhibited or activated by hypotonic cell swelling or hypertonic cell shrinkage. A recent study also demonstrated an alteration of ion transport across the human airway epithelium by hypertonic saline, independently of the altered osmolarity [36, 46]. It was found that hyperosmolarity of the mucosal airway surface liquid reduced the transepithelial voltage of the nasal epithelium [36, 46]. Exposure to hyperosmolarity also affected other functions of the airway epithelium like relaxation and contraction of the tracheal smooth muscle [15, 18]. Most importantly, hypertonic saline treatment of the airways has a mucolytic effect, improves mucociliary clearance and is frequently used as a treatment in asthma or cystic fibrosis [8, 43]. Thus, improved lung function, such as increase in forced expiratory volume in 1 s and forced vital capacity have been reported, along with other beneficial effects [43]. Treatment with nebulized hypertonic saline caused enhanced mucociliary clearance and improved lung function despite its negative impact on ciliary beat frequency [4]. Thus, other factors may essentially contribute to the beneficial effects of hypertonic saline on lung function. Most importantly, hypertonic saline on the luminal side of the respiratory epithelium was shown to slow down Na^+ absorption [46]. We therefore examined in the present study i) if reduced Na^+ absorption could be due to osmotic inhibition of epithelial Na^+ channels and ii) if the inhibitory effects on ENaC by purinergic stimulation and activation of CFTR are due to osmotic cell swelling or shrinkage.

Materials and Methods

cRNAs for ENaC and CFTR and Expression in *Xenopus* Oocytes

cDNA encoding rat α,β,γ ENaC (kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland), and human CFTR were linearized in pBluescript or pTLN [19] with *Not*I or *Mlu*I, and in vitro transcribed using T7, T3 or SP6 promoter and polymerase (Message Machine, Ambion, USA). Isolation and microinjection of oocytes have been described in a previous report [32]. In brief, after isolation from adult *Xenopus laevis* female frogs (*Xenopus* Express, South Africa), oocytes were dispersed and defolliculated by a 45 min treatment with collagenase (type A, Boehringer, Germany). Subsequently, oocytes were rinsed and kept at 18°C in ND96 buffer (in mmol/L): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55), supplemented with theophylline (0.5 mmol/L) and gentamycin (5 mg/L).

Double-Electrode Voltage Clamp

Oocytes were injected with cRNA (1–10 ng) after dissolving in about 50 nL double-distilled water (Nanoliter Injector WPI, Germany). Water-injected oocytes served as controls. 2–4 days after injection, oocytes were impaled with two electrodes (Clark Instruments) which had a resistances of < 1 MΩ when filled with 2.7 mol/L KCl. Two bath electrodes were used with resistances of 1.7 and 2.2 kΩ, respectively, when immersed in ND96 bath solution. Using two bath electrodes and a virtual-ground headstage, the voltage drop across R_{serial} was effectively zero. Membrane currents were measured by voltage clamping of the oocytes (Warner Oocyte Clamp Amplifier OC725C) in intervals from −90 mV to +30 mV, in steps of 10 mV, each 1 s. Current data were filtered at 50 Hz. Data were collected continuously (PowerLab, AD-lnstruments, Australia) and were analyzed using the programs Chart and Scope (PowerLab, AD-lnstruments, Australia). Conductances were calculated according to Ohm's law and amiloride-sensitive conductances (G_{Amil}) were used in the present report to express the amount of whole-cell conductance that is inhibited by 10 μmol/L amiloride. During the whole experiment, the bath was continuously perfused at a rate of 5–10 mL/min. All experiments were conducted at room temperature (22°C).

Ussing Chamber Experiments

Tracheas were taken from mice (Quackenbush, animal facility of the University of Queensland) after sacrificing the animals by cervical dislocation, and opened by a longitudinal cut after all connective tissues had been removed. Mouse distal colon was removed from the animal and the mucosa was separated mechanically from the submucosal tissue. Tissues were put immediately into a cold buffer solution of the following composition (mmol/L): NaCl 145, KCl 3.8, D-glucose 5, $MgCl_2$ 1, HEPES 5, Ca-gluconate 1.3. The tissues were mounted into a modified Ussing chamber with a circular aperture of 0.95 mm^2. The luminal and basolateral sides of the epithelium were perfused continuously at a rate of 10 mL/min (chamber volume 2 mL). The bath solution had the following composition (mmol/L): NaCl 145, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, HEPES 5, Ca-gluconate 1.3. pH was adjusted to 7.4. Bath solutions were heated to 37°C using a water jacket. Experiments were carried out under open-circuit conditions. Values for transepithelial voltages (V_{te}) were referred to the serosal side of the epithelium. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses ($\Delta I = 0.5$ μA). Voltage deflections obtained under conditions without the mucosa present in the chamber were subtracted from those obtained in the presence of the tissues. R_{te} was calculated according to Ohm's law ($R_{te} = \Delta V_{te}/\Delta I$). The equivalent short-circuit current (I_{sc}) was calculated ($I_{sc} = V_{te}/R_{te}$) and the amiloride-sensitive I_{sc} ($I_{sc\text{-}Amil}$) is used to express the amount of equivalent short-circuit current that is inhibited by 10 μmol/L amiloride. Tissue preparations were only accepted if the transepithelial resistance exceeded that obtained for

an empty chamber at least by a factor of 3. Recordings were usually stable for 3 to 4 hours.

Materials and Statistical Analysis

For the experiments with *Xenopus* oocytes a hypertonic ND96 bath solution (310 mosmol/L) was used that was obtained by adding 120 mmol/L mannitol to the normotonic (190 mosmol/L) ND96 solution. Hypotonic bath solution (70 mosmol/L) was prepared by removing 120 mmol/L mannitol from a modified isotonic ND96 solution, in which 60 mmol/L NaCl had been replaced by 120 mmol/L mannitol. For the Ussing chamber experiments a hypertonic bath solution (360 mosmol/L) was prepared by adding either 80 mmol/L mannitol or 40 mmol/L NaCl to the normotonic (280 mosmol/L) Ringer solution. Hypotonic bath solution (200 mosmol/L) was obtained by removing 80 mmol/L mannitol from a modified isotonic bath solution in which 40 mmol/L NaCl had been replaced by 80 mmol/L mannitol. All solutions were checked for proper osmolality (mosmol/kg) using a 5500 vapor pressure osmometer (Wescor, Australia) and are expressed as osmolarities (mosmol/L). Effects of amiloride in trachea and oocytes were examined typically ≥10 min after exposure to the hypertonic or hypotonic solutions. All used compounds were of highest available grade of purity. Adenosine 5′-triphosphate (ATP), uridine 5′-triphosphate (UTP), 3-isobutyl-1-methylxanthine (IBMX), forskolin, tyrphostin and mannitol were all from Sigma (Australia). Student's *t*-test p values < 0.05 were accepted to indicate statistical significance, with number n of experiments given in parentheses.

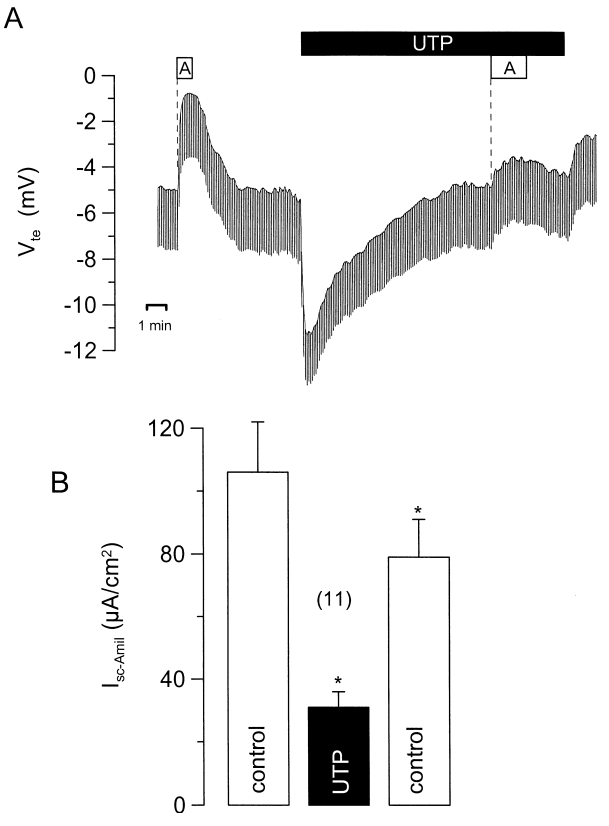

Fig. 1. Inhibition of Na^+ transport by UTP in mouse trachea. (*A*) Continuous recording of the transepithelial voltage (V_{te}) in a perfused micro-Ussing chamber and effects of UTP and amiloride (*A*; 10 μmol/L) in the absence or presence of UTP (100 μmol/L). (*B*) Summary of the amiloride-sensitive short-circuit currents ($I_{sc\text{-}Amil}$) before, during and 60 min after stimulation with UTP. *Significant inhibition of $I_{sc\text{-}Amil}$ by UTP and recovery from inhibition (paired *t*-tests); *n*, number of experiments.

Results

Purinergic Stimulation and Activation of CFTR Inhibits Amiloride-sensitive Na^+ Transport in Mouse Airways and Colon

Previous reports have shown that activation of purinergic receptors in human and mouse airways leads to activation of a Ca^{2+}-dependent Cl^- conductance and reduces amiloride-sensitive Na^+ absorption [25, 33]. In the present Ussing chamber experiments we also found that stimulation of mouse trachea with 100 μmol/L UTP (uridine 5′-triphosphate) induced a rapid voltage deflection and increase in lumen-negative I_{sc} from -114.4 ± 22.1 to -248.3 ± 28.6 μA/cm^2 ($I_{sc\text{-}UTP} = 134.8 \pm 18.3$ μA/cm^2; $n = 11$). Transient increase in Cl^- secretion was paralleled by an inhibition of amiloride-sensitive Na^+ absorption ($I_{sc\text{-}Amil}$) of 74.3 ± 8.9 μA/cm^2 ($n = 11$), indicating inhibition of epithelial Na^+ channels by purinergic stimulation (Fig. 1). Inhibition of ENaC was reversible within 1 h after washout of UTP (Fig. 1*B*). In the presence of low extracellular Cl^- (luminal and basolateral 5 mmol/L), activation of Cl^- secretion ($I_{sc\text{-}UTP}$) and inhibition of ($I_{sc\text{-}Amil}$) were both significantly reduced to 34.2 ± 5.8 μA/cm^2 and 32.4 ± 6.7 μA/cm^2 ($n = 4$), respectively, similar to what has been reported previously [25].

Amiloride-sensitive Na^+ absorption is also inhibited by CFTR. This, however, cannot be demonstrated in mouse trachea, since the level of expression of CFTR in this tissue is very low [40]. We therefore examined regulation of ENaC by CFTR in the mouse colon, where CFTR expression is abundant. As shown in Fig. 2, stimulation of this tissue with IBMX (100 μmol/L) and forskolin (2 μmol/L) activates CFTR and induces Cl^- secretion ($I_{sc\text{-}CFTR} = 118.8 \pm 22.8$ μA/cm^2, $n = 14$). This is paralleled by a suppression of the effects of amiloride (10 μmol/L_) on V_{te} and inhibition of $I_{sc\text{-}Amil}$ (from -27.9 ± 6.2 to -14.3 ± 5.0 μA/cm^2; $n = 14$). As described for the effects of UTP on Cl^- secretion and inhibition of $I_{sc\text{-}Amil}$, in the presence of low extracellular Cl^- (luminal and basolateral 5 mmol/L), activation of Cl^- secretion by IBMX/forskolin ($I_{sc\text{-}CFTR}$) was completely abolished and $I_{sc\text{-}Amil}$ was indistinguishable before (11.0 ± 2.5 μA/cm^2) and after (10.7 ± 2.7 μA/cm^2; $n = 5$) stimulation. Thus, both purinergic and CFTR-dependent Cl^- transport and inhibition of Na^+ transport are Cl^- dependent.

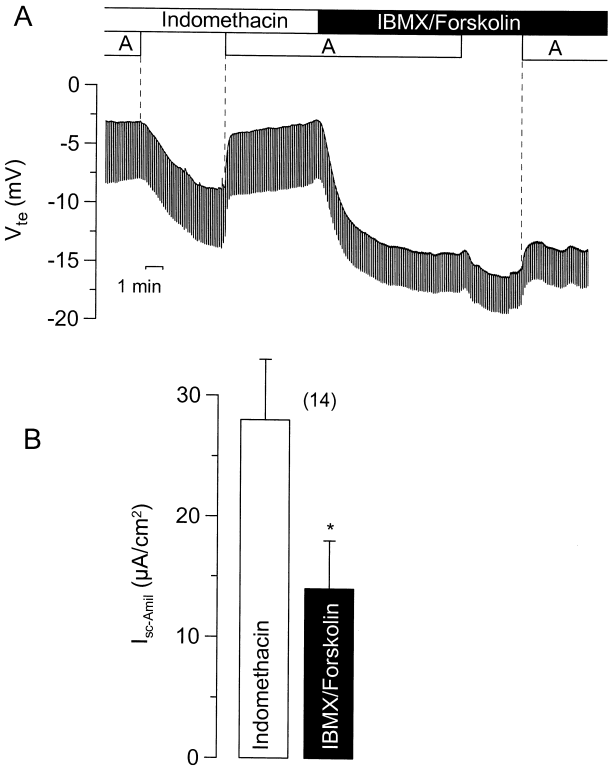

Fig. 2. Inhibition of Na^+ transport by activation of CFTR in mouse colon. (*A*) Continuous recording of the transepithelial voltage (V_{te}) in a perfused micro-Ussing chamber and effects of amiloride (*A*; 10 μmol/L) in the presence of 10 μmol/L indomethacin (low intracellular cAMP, inactive CFTR) or 100 μmol/l IBMX/ 2 μmol/L forskolin (high intracellular cAMP, active CFTR). (*B*) Summary of the amiloride-sensitive short-circuit currents ($I_{sc-Amil}$) before (Indomethacin) and after (IBMX/Forskolin) activation of CFTR. *Significant inhibition of $I_{sc-Amil}$ by stimulation of CFTR (paired *t*-test).

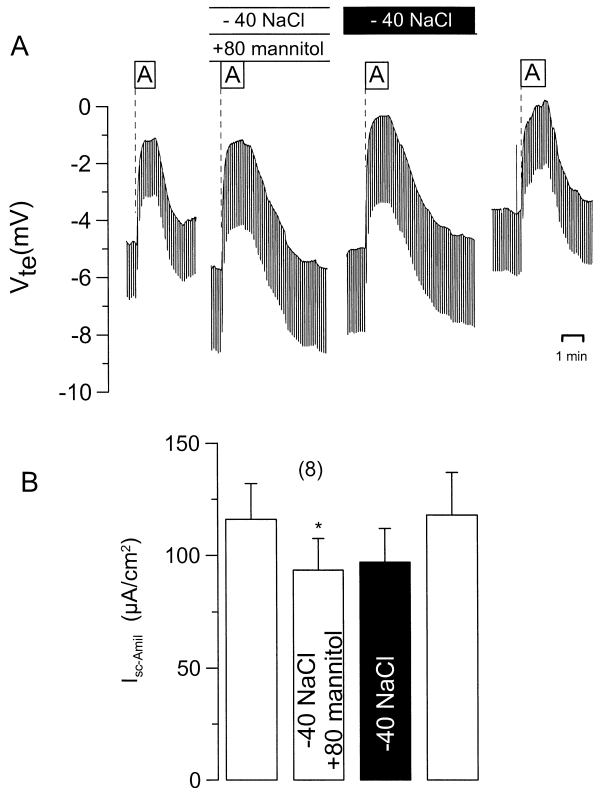

Fig. 3. Effect of hypotonic bath solution on amiloride-sensitive transport in mouse trachea. (*A*) Continuous recording of the transepithelial voltage (V_{te}) in a perfused micro-Ussing chamber and effects of amiloride (*A*; 10 μmol/L) under control conditions, after isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol and after removal of 80 mmol/L mannitol (hypotonic bath solution). (*B*) Summary of the amiloride-sensitive short-circuit currents ($I_{sc-Amil}$) under control conditions (pre-control), after isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol, after removal of 80 mmol/L mannitol (−40 NaCl; hypotonic bath solution) and under post-control conditions. *Significant inhibition of $I_{sc-Amil}$ after isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol (paired *t*-test).

Effects of Hypotonic and Hypertonic Stress on Amiloride-sensitive Na^+ Transport in Mouse Trachea

To examine if purinergic and CFTR-mediated inhibition of epithelial Na^+ absorption is due to changes in cell volume, we investigated the effects of cell swelling and cell shrinkage on amiloride-sensitive Na^+ absorption. To that end, mouse tracheas were mounted in perfused Ussing chambers and were exposed on both sides to hypotonic (200 mosmol/L) or hypertonic (360 mosmol/L) bath solutions. As shown in Fig. 3, isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol reduced amiloride-sensitive Na^+ transport, which is due to removal of charge carriers. However, removal of mannitol and thus cell swelling did not change the effects of amiloride on V_{te} and did not affect $I_{sc-Amil}$. In contrast, when cell shrinkage was induced by either adding 80 mmol/L mannitol or 40 mmol/L NaCl, $I_{sc-Amil}$ was reversibly inhibited by almost 50% (Fig. 4). Inhibition of $I_{sc-Amil}$ by hypertonic bath solution was similar in the presence of a low (5 mmol/L) extracellular Cl^- concentration: $I_{sc-Amil}$ changed from 121.2 ± 12.4 μA/cm² (normotonic) to 37.3 ± 4.7 μA/cm² (hypertonic) (n = 6). Taken together, these data indicate inhibition of amiloride-sensitive Na^+ absorption in mouse trachea by hypertonic bath solution, which may be due to inhibition of ENaC by cell shrinkage, while hypotonic cell swelling does not affect Na^+ transport.

Similar changes in cell volume may occur during activation of CFTR or stimulation of purinergic receptors by ATP or UTP and induction of Cl^- secretion. We therefore examined the effects of ATP on Na^+ transport in mouse trachea in the presence of hypertonic or hypotonic bath solutions. As summarized in Fig. 5*A*, $I_{sc-Amil}$ is inhibited by hypertonic bath solution, similar to the experiments shown in

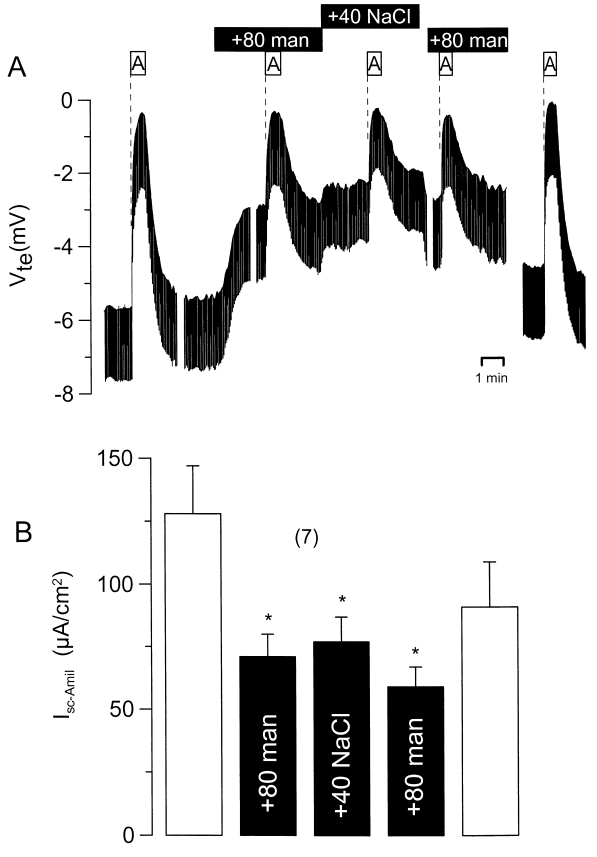

Fig. 4. Effect of hypertonic bath solution on amiloride-sensitive Na^+ transport in mouse trachea. (*A*) Continuous recording of the transepithelial voltage (V_{te}) in a perfused micro-Ussing chamber and effects of amiloride (*A*; 10 μmol/L) under control conditions and after exposure to hypertonic bath solutions containing either additional 80 mmol/L mannitol or 40 mmol/L NaCl. (*B*) Summary of the amiloride-sensitive short-circuit current ($I_{sc\text{-}Amil}$) under control conditions (pre-control), after adding 80 mmol/L mannitol (+80 man) or 40 mmol/L NaCl (+40 NaCl; hypertonic bath solution) and under post-control conditions. *Significant inhibition of $I_{sc\text{-}Amil}$ by the hypertonic bath solutions (paired *t*-test).

Fig. 4. Subsequent stimulation of the epithelium with 100 μmol/L ATP further reduced $I_{sc\text{-}Amil}$, with a fractional inhibition of $I_{sc\text{-}Amil}$ (65%) that was not different from the fractional inhibition of $I_{sc\text{-}Amil}$ in isotonic buffer (71%). In contrast, when tracheas were exposed to hypotonic bath solution, $I_{sc\text{-}Amil}$ was not affected and the inhibitory effect of ATP on $I_{sc\text{-}Amil}$ was still detectable, although significantly reduced (18%), when compared to the effects of ATP on $I_{sc\text{-}Amil}$ in the presence of a normotonic solution (71%) (Figs. 1 and 5*B*). These data suggest an additive inhibitory effect on Na^+ absorption of hyperosmotic bath solution and purinergic stimulation in mouse trachea and indicate that cell swelling somehow counteracts the inhibitory effect of ATP on $I_{sc\text{-}Amil}$.

Inhibitory Effects of Purinergic Stimulation on Amiloride-sensitive Na^+ Transport in Mouse Colon

Purinergic inhibition of amiloride-sensitive Na^+ transport was also examined in mouse colon. As described above, stimulation of CFTR inhibits Na^+ absorption in this tissue. In order to be able to examine the impact of luminal application of ATP on amiloride-inhibitable Na^+ absorption, we inactivated CFTR by incubating the tissue with 10 μM indomethacin, a cyclooxygenase inhibitor that blocks endogenous production of cAMP and thus inactivates CFTR [21, 23, 30]. In the presence of indomethacin and due to luminal application of ATP (100 μM), the lumen-negative I_{sc} of -59.2 μA/cm² was transiently reduced to -44.79 μA/cm² ($n = 5$), which is probably due to transient activation of luminal K^+ channels [23, 30, 47]. This was paralleled by significant inhibition of $I_{sc\text{-}Amil}$ from 21 ± 8.1 μA/cm² to 10.7 ± 3.8 μA/cm² ($n = 5$). In analogy to the experiments described for mouse trachea, we examined the effects of ATP on Na^+ transport in mouse colon in the presence of hypertonic or hypotonic bath solutions. The results were similar to those obtained for mouse trachea and are summarized in Fig. 5*C*, and *D*: $I_{sc\text{-}Amil}$ was significantly inhibited by hypertonic bath solution. Subsequent stimulation of the epithelium with 100 μmol/L ATP further reduced $I_{sc\text{-}Amil}$ by 57%, which was not different from the inhibition in isotonic buffer (50%). However, when exposed to hypotonic bath solution, $I_{sc\text{-}Amil}$ was not affected and the inhibitory effect of ATP on $I_{sc\text{-}Amil}$ was reduced (30%), when compared to normotonic solution (50%) (Fig. 5*C*, and *D*). Finally, when extracellular Cl^- was replaced by gluconate, $I_{sc\text{-}Amil}$ was reduced from 41.8 ± 15 to 18.5 ± 5.1 μA/cm² ($n = 4$) and application of ATP further inhibited $I_{sc\text{-}Amil}$ to 12.9 ± 3.3 μA/cm².

Effects of Osmotic Stress on ENaC in *Xenopus* Oocytes

The present results may be due to direct osmotic effects on the epithelial Na^+ channel ENaC. We therefore expressed ENaC in *Xenopus* oocytes, which were then exposed to hypertonic (310 mosmol/L) or hypotonic (70 mosmol/L) media. Ion currents were measured by voltage-clamping the oocytes from −90 to +30 mV under control conditions and after exposure to hypertonic (+120 mmol/L mannitol) or hypotonic (−60 mmol/L NaCl) bath solution. Fig. 6 demonstrates the inhibitory effects of amiloride (10 μmol/L), which were detectable under all three experimental conditions. In contrast, hypotonic or hypertonic stress in water-injected control oocytes did not generate any amiloride-sensitive whole-cell currents and had no significant effects on whole-cell currents within the first 15 min after application of the

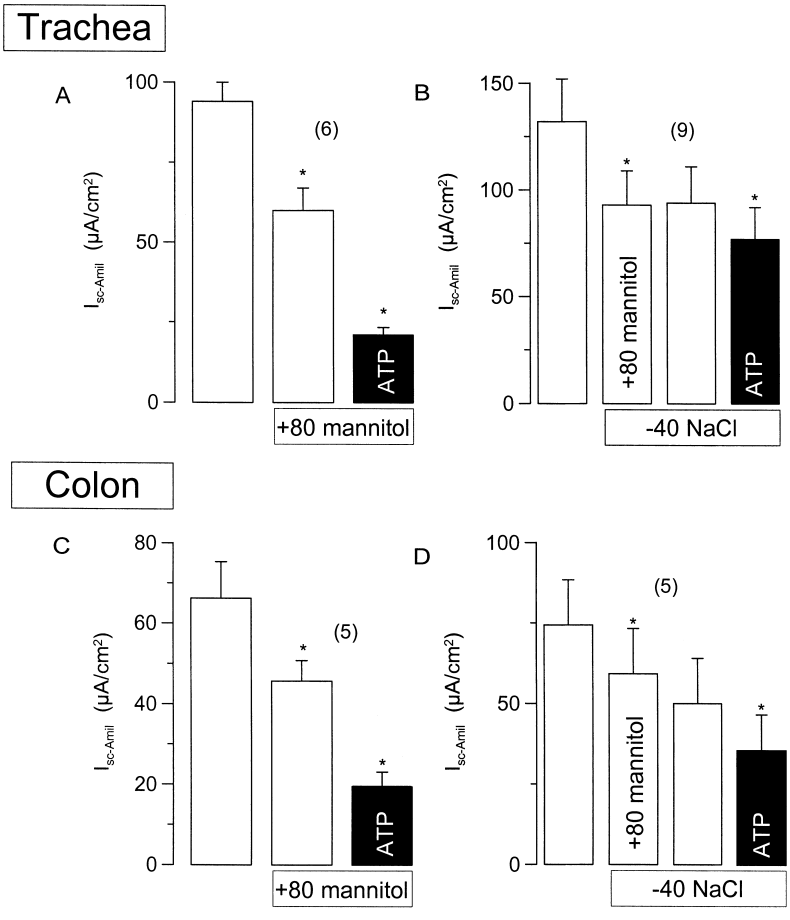

Fig. 5. Effect of osmotic stress and stimulation with ATP on amiloride-sensitive transport in mouse trachea and colon. (*A*) Summary of the amiloride-sensitive short-circuit current ($I_{sc\text{-}Amil}$) in mouse trachea under control conditions, after adding 80 mmol/L mannitol (hypertonic bath solution) and after applying 100 μmol/L ATP to a hypertonic bath solution. (*B*) Summary of $I_{sc\text{-}Amil}$ in mouse trachea under control conditions, after isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol, after removal of 80 mmol/L mannitol (hypotonic bath solution) and after applying 100 μmol/L ATP to a hypotonic bath solution. (*C*) Summary of $I_{sc\text{-}Amil}$ in mouse colon under control conditions, after adding 80 mmol/L mannitol (hypertonic bath solution) and after applying 100 μmol/L ATP to a hypertonic bath solution. (*D*) Summary of $I_{sc\text{-}Amil}$ in mouse trachea under control conditions, after isotonic replacement of 40 mmol/L NaCl by 80 mmol/L mannitol, after removal of 80 mmol/L mannitol (hypotonic bath solution) and after applying 100 μmol/L ATP to a hypotonic bath solution. *Significant inhibition of $I_{sc\text{-}Amil}$ by hypertonic bath solution, isotonic replacement of NaCl by mannitol and additional stimulation with ATP (paired *t*-tests).

osmotic stress ($n = 5$, *data not shown*). Moreover, we examined the possible impact of osmotic stress on amiloride-sensitive Na^+ conductance (G_{Amil}) in the presence of a high (101 mmol/L) or low (5 mmol/L) extracellular Cl^- concentration. The data from these experiments are summarized in Fig. 7 and indicate that except for a slight increase of G_{Amil} by hypotonic bath solution in the presence of high extracellular Cl^-, there were no significant inhibitory effects of either hypertonic or hypotonic stress on G_{Amil}. This result is clearly different from the results of another study [17] but confirms other previously published data [2].

Inhibition of ENaC by CFTR in the Presence of Hypertonic or Hypotonic Bath Solution

ENaC is inhibited in *Xenopus* oocytes upon activation of coexpressed CFTR [22, 32]. Inhibition of amiloride-sensitive Na^+ currents by activation of CFTR with 1 mmol/L IBMX and 2 μmol/L forskolin is shown in Fig. 8*A*. Inhibition of ENaC was reversible upon removal of Cl^- from the extracellular bath solution (5 Cl^-), confirming previous results [6, 22] (Fig. 8*A*, and *B*). In order to examine if osmotic stress interferes with the inhibitory effects of CFTR on ENaC, CFTR was activated in the presence of either hypertonic or hypotonic bath solution. The data from these experiments, which are summarized in Fig. 8*C* and *D*, clearly indicate that CFTR-mediated inhibition of ENaC was present under both hypertonic or hypotonic conditions. Although protein tyrosine kinases (PTK) have been shown to be activated during osmotic stress in several tissues [13], inhibition of ENaC by CFTR was not different in oocytes injected with the PTK inhibitor tyrphostin B48 (final concentration 250 μmol/L) (*data not shown*). Taken together, the present data do not support a role of volume changes during CFTR or purinergic inhibition of ENaC or amiloride-sensitive Na^+ absorption, but supply evidence that changes in the intracellular Cl^- concentration are in charge of the inhibitory effects on Na^+ transport.

Discussion

Volume Regulation and Mechanosensitivity of ENaC

Conflicting results have been reported on the effects of hydrostatic pressure, cell shrinkage or cell swelling on ENaC. When expressed in *Xenopus* oocytes,

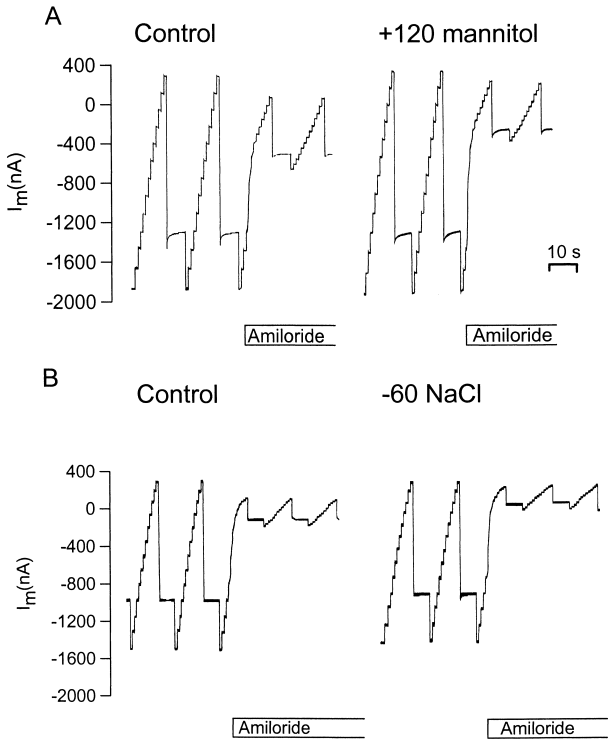

Fig. 6. Original recordings of whole-cell currents in ENaC-expressing oocytes and inhibition by amiloride (10 μmol/L) under control conditions and in the presence of a hypertonic (+120 mmol/L mannitol) or hypotonic (−60 mmol/L NaCl) bath solution. Whole-cell currents were measured during continuous voltage clamp from −90 mV to +30 mV in steps of 10 mV.

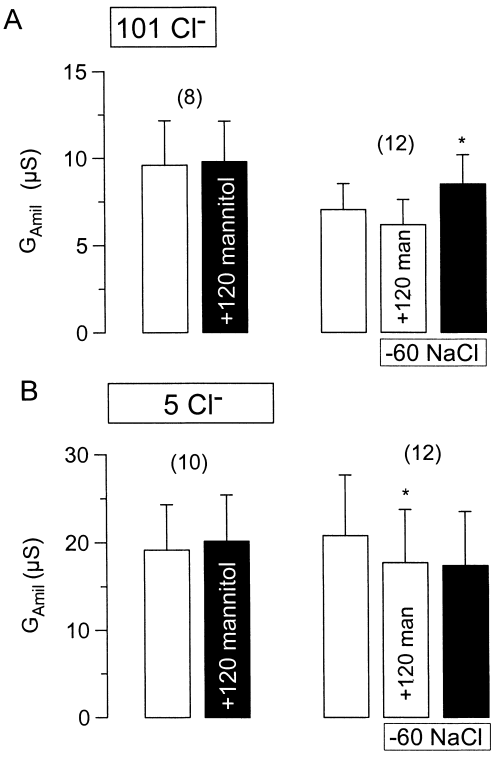

Fig. 7. Summary of the calculated amiloride-sensitive whole-cell conductances (G_{Amil}) under control conditions and after application of osmotic stress. (*A*) Effects of hyperosmotic (+120 mmol/L mannitol) and hyposmotic (−60 mmol/L NaCl) stress on G_{Amil} in the presence of a high (101 mmol/L) extracellular Cl^- concentration. (*B*) Effects of hyperosmotic (+120 mmol/L mannitol) and hyposmotic (−60 mmol/L NaCl) stress on G_{Amil} in the presence of a low (5 mmol/L) extracellular Cl^- concentration. *Significant difference from control (paired *t*-test).

ENaC was either inhibited [17], slightly activated (*present study*) or was not affected [2] by hypotonic cell swelling. Hypertonic cell shrinkage either activated [17] or inhibited [2] Na^+ conductance. Application of hydrostatic pressure had variable effects on the gating of native apical Na^+ channels in cortical collecting duct cells [38] or activated Na^+ channels reconstituted into planar lipid bilayers [1]. In epithelial tissues, Na^+ conductance was either activated in frog skin, hepatocytes and fetal distal lung epithelium [7, 37, 45] or was inhibited in A6 cells [34]. Moreover, and in addition to the above described results, a flow-dependent regulation of ENaC has been found, which may be an important regulator of Na^+ channel activity in the cortical collecting duct [41]. Given the differences among all the experimental setups and experimental protocols used in the different studies, it is not completely surprising that discrepant results have been reported. In agreement with a previous report [2], we would conclude from our studies that ENaC has no or only a minor mechanosensitivity and may rather be regulated by changes in the intracellular ion concentration, in particular $[Cl^-]_i$, which occur upon application of osmotic stress. Accordingly, G_{Amil} was generally higher in oocytes adapted to low extracellular Cl^-, and hypotonic cell swelling (and concomitant decrease in $[Cl^-]_i$) enhanced G_{Amil} only in the presence of high (101 mmol/L) extracellular Cl^-. Along this line, the present data supply further evidence that control of ENaC by purinergic stimulation and CFTR is not due to osmotic stress but is caused by a change in $[Cl^-]_i$. These results are reminiscent of the Cl^- feedback regulation described for mouse salivary duct cells [11, 12]. In those studies, both Cl^-- and Na^+-sensitive G proteins were shown to mediate the inhibitory signal for ENaC [10, 20].

The present results demonstrate inhibition of amiloride-sensitive transport in mouse airways. A previous study on human airway epithelia using transepithelial measurements, intracellular microelectrodes and quantitative microscopy detected an increase in $[Cl^-]_i$ during increase in luminal osmolarity along with inhibition of Na^+ absorption [46]. Reduced airway Na^+ absorption by hyperosmolarity was caused by several events, including inhibition of luminal ENaC and basolateral K^+ conductance along with an increase in the paracellular shunt resistance [46]. The authors suggested an osmotic sensor that is transducing selective responses to airway

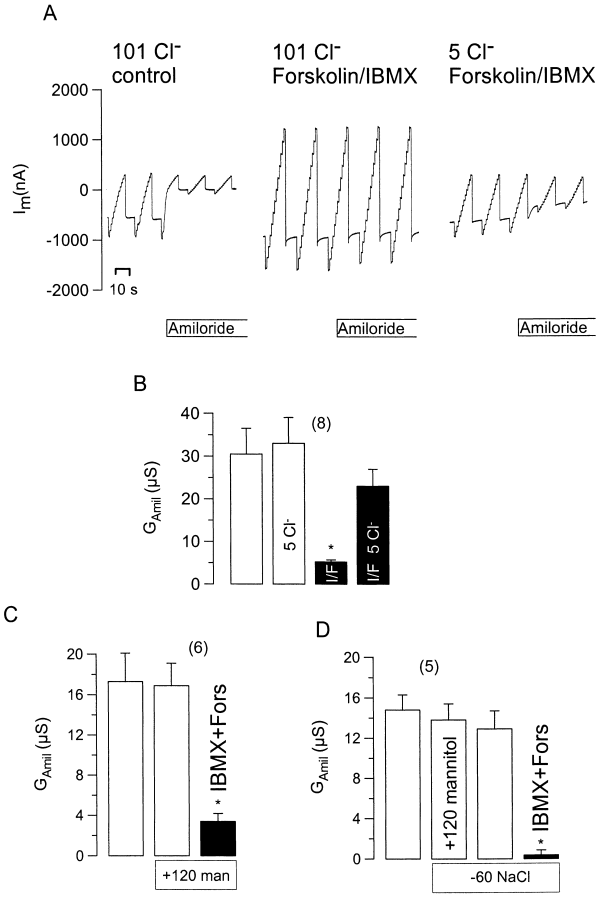

Fig. 8. Inhibition of ENaC by CFTR in *Xenopus* oocytes. (*A*) Original recordings of whole-cell currents in ENaC- and CFTR-coexpressing *Xenopus* oocytes and effects of amiloride (10 μmol/L) under control conditions, after activation of CFTR by forskolin (10 μmol/L) and IBMX (1 mmol/L) and after replacing extracellular Cl^- by gluconate (5 Cl^-) in the presence of forskolin and IBMX. (*B*) Summary of the calculated amiloride-sensitive whole-cell conductances (G_{Amil}) under control conditions and after stimulation of CFTR with IBMX and forskolin (*I*/*F*) and effect of low extracellular Cl^-. (*C*) Summary of G_{Amil} under control conditions and in a hypertonic (+120 mmol/L mannitol) extracellular bath solution and inhibition of G_{Amil} by CFTR under hypertonic stress. (*D*) Summary of G_{Amil} under control conditions, after isotonic replacement of 60 mmol/L NaCl by 120 mmol/L mannitol in the extracellular bath solution and in a hypotonic (−60 mmol/L NaCl) bath solution, and inhibition of G_{Amil} by CFTR under hypotonic stress. *Significant difference from control (paired *t*-test).

epithelial cells. According to the present results and to data obtained on the mouse salivary duct (12, 20), $[Cl^-]_i$ and/or $[Na^+]_i$ could serve as such a transducer, controlling the rate of luminal Na^+ entry. The feedback between the airway surface liquid (ASL) and airway epithelia actively controls the ASL volume by regulating the rate of ion transport and volume absorption [44]. The results imply the use of hypertonic saline in the airways of CF patients, in order to reduce excessive Na^+ transport and hyperabsorption of electrolytes.

In fact, hypertonic saline was shown to alter ion transport in the human airway epithelium [36, 46] and treatment of CF airways with aerosolized hypertonic saline improved mucociliary clearance in CF patients and normal subjects. No differences were found when the effects of saline were compared with those of amiloride [35]. In good agreement with the results from the present study, human airways in vivo respond to topical application of hypertonic saline or mannitol solution [36, 39]. Thus, treatment of CF airways with hypertonic saline offers a cost-effective and efficient way of improving mucociliary clearance and lung function in CF [8, 43]. The positive impact of hypertonic saline or mannitol solution can be explained by both improved rheology of the mucus and attenuation of amiloride-sensitive hyperabsorption of NaCl, eventually due to an increase in the intracellular Cl^- concentration.

An increase in the intracellular Cl^- concentration is also likely to play a role in inhibition of Na^+ absorption during activation of CFTR in mouse colon or stimulation of purinergic receptors in mouse trachea. As shown in the present experiments, CFTR does not inhibit ENaC at a low extracellular Cl^- concentration and inhibition of ENaC by CFTR in *Xenopus* oocytes is reversible upon replacement of extracellular Cl^- by gluconate. Moreover, inhibition of Na^+ absorption by puringeric stimulation is augmented in the presence of high extracellular Cl^- [25]. In *Xenopus* oocytes ENaC is not only inhibited by CFTR, but also by other Cl^- conductances such as CIC-0 and CIC-2 [14, 22]. In *Xenopus* oocytes coexpressing ENaC and CFTR or CIC-0, $[Cl^-]_i$ enhanced in the presence of a high extracellular Cl^- concentration. Similar changes may happen in colonic and airway epithelial cells, which absorb Na^+ under baseline conditions, but activate luminal Cl^- channels and Cl^- uptake by basolateral $Na^+/2Cl^-/K^+$ cotransporters during cAMP-dependent stimulation or when challenged by purinergic agonists. While these changes are likely to cause osmotic stress for epithelial cells, the present results suggest that inhibition of Na^+ absorption by CFTR or purinergic agonists are due to changes in $[Cl^-]_i$ rather than changes in cell volume.

This work was supported by DFG Ku756, Cystic Fibrosis Australia and ARC grant ARC A00104609.

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