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# No evidence for inhibition of ENaC through CFTR-mediated release of ATP

Jens König [a], R. Schreiber [a], M. Mall [b], K. Kunzelmann [a,*]

[a] *Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia*
[b] *Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, The University of North Carolina at Chapel Hill, 7011 Thurston Bowles Building, Chapel Hill, NC 27599-7248, USA*

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**Abstract**

Both purinergic stimulation and activation of cystic fibrosis transmembrane conductance regulator (CFTR) increases $Cl^-$ secretion and inhibit amiloride-sensitive $Na^+$ transport. CFTR has been suggested to conduct adenosine 5′-triphosphate (ATP) or to control ATP release to the luminal side of epithelial tissues. Therefore, a possible mechanism on how CFTR controls the activity of epithelial $Na^+$ channels (ENaC) could be by release of ATP or uridine 5′-triphosphate (UTP), which would then bind to P2Y receptors and inhibit ENaC. We examined this question in native tissues from airways and colon and in *Xenopus* oocytes. Inhibition of amiloride-sensitive transport by both CFTR and extracellular nucleotides was observed in colon and trachea. However, nucleotides did not inhibit ENaC in *Xenopus* oocytes, even after coexpression of $P2Y_2$ receptors. Using different tools such as hexokinase, the P2Y inhibitor suramin or the $Cl^-$ channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), we did not detect any role of a putative ATP secretion in activation of $Cl^-$ transport or inhibition of amiloride sensitive short circuit currents by CFTR. In addition, $N^2$,2′-*O*-dibutyrylguanosine 3′,5′-cyclic monophosphate (cGMP) and protein kinase G (PKG)-dependent phosphorylation or the nucleoside diphosphate kinase (NDPK) do not seem to play a role for the inhibition of ENaC by CFTR, which, however, requires the presence of extracellular $Cl^-$.


*Keywords:* CFTR; ENaC; *Xenopus* oocytes; Trachea; Colon; Cystic fibrosis; ATP; UTP; Purinergic receptor; Epithelial transport

## 1. Introduction

Amiloride-sensitive epithelial $Na^+$ channels (ENaC) are essential for electrogenic absorption of $Na^+$ in kidney, airways, intestine and other epithelial tissues [6]. ENaC is expressed in airway and colonic epithelial cells, together with cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channels [18]. CFTR is not only a $Cl^-$ channel but it also controls the activity of numerous other ion transport proteins [15,35]. It has been demonstrated that ENaC is inhibited by CFTR in airway and colonic epithelial tissues and cells coexpressing both proteins recombinantly [19,21]. Others have been unable to demonstrate inhibition of ENaC by CFTR in *Xenopus* oocytes or demonstrated activation of ENaC by CFTR in the sweat duct epithelium. These differences are likely due to variable relative expression levels of CFTR and ENaC in *Xenopus* oocytes and indicate functional differences between the different epithelial tissues, respectively [14]. We have postulated that activation of CFTR $Cl^-$ channels and simultaneous inhibition of ENaC results in a switch of epithelial ion transport from absorption under control conditions, towards secretion after stimulation by secretagogues [15,18].

In a surprising similarity to the regulation of ENaC by CFTR, an inverse regulation of $Cl^-$ and $Na^+$ transport has been found for the stimulation of purinergic P2Y receptors, which are colocalized in respiratory epithelial cells together with ENaC [2,27,32]. Binding of adenosine 5′-triphosphate (ATP) or uridine 5′-triphosphate (UTP) to P2Y receptors leads to an increase in intracellular $Ca^{2+}$ and

*Abbreviations:* CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-1-methylxanthine; cAMP, 5′ cyclic adenosine monophosphate; ENaC, epithelial $Na^+$ channel; UTP, uridine 5′-triphosphate; ATP, adenosine 5′-triphosphate; PKG, protein kinase G; cGMP, $N^2$,2′-*O*-dibutyrylguanosine 3′,5′-cyclic monophosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; PKG (peptide) inhibitor, H-Arg-Lys-Arg-Ala-Arg-Lys-glu-OH

* Corresponding author. Tel.: +61-7-3365-4104; fax: +61-7-3365-1766.
*E-mail addresses:* mmall@med.unc.edu (M. Mall), uqkkunze@mailbox.uq.edu.au (K. Kunzelmann).

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activation of $Ca^{2+}$-dependent $Cl^-$ channels (CaCC) [27]. In parallel, purinergic agonists attenuate amiloride-sensitive $Na^+$ transport, probably by inhibition of ENaCs [2,27,32]. For both CFTR and purinergic inhibition of ENaC, the underlying mechanisms have not yet been described in detail [17]. Some requirements have been identified, such as a functional first nucleotide binding domain (CFTR) or G protein function (P2Y receptor) [16,20]. Along this line, $Cl^-$ transport induced by CFTR $Cl^-$ channels or $P2Y_2$-activated CaCC channels plays a crucial role [14,20]. These similarities suggest a common link for CFTR and purinergic inhibition of ENaC. Such a link could exist through the control of ATP transport by CFTR [11,36,37]. ATP transport by CFTR, however, is a controversial issue. It has been detected in some but not in other studies [7,8,22,23,33,38,43]. ATP transport by CFTR or control of an ATP transporting protein by CFTR [1] should lead to 5′ cyclic adenosine monophosphate (cAMP)-mediated release of ATP or UTP, stimulation of $Ca^{2+}$-activated $Cl^-$ channels and inhibition of ENaC. We examined this possibility and other ATP-dependent regulatory pathways in the present study. We found no evidence for a CFTR-mediated release of purinergic agonists and a functional link of both purinergic and CFTR-dependent inhibition of ENaC via the proposed mechanism.

## 2. Methods

### 2.1. cRNAs for ENaC, CFTR, $P2Y_2$ and NDPK and expression in Xenopus oocytes

cDNA encoding rat (α,β,γ) ENaC (kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lau-

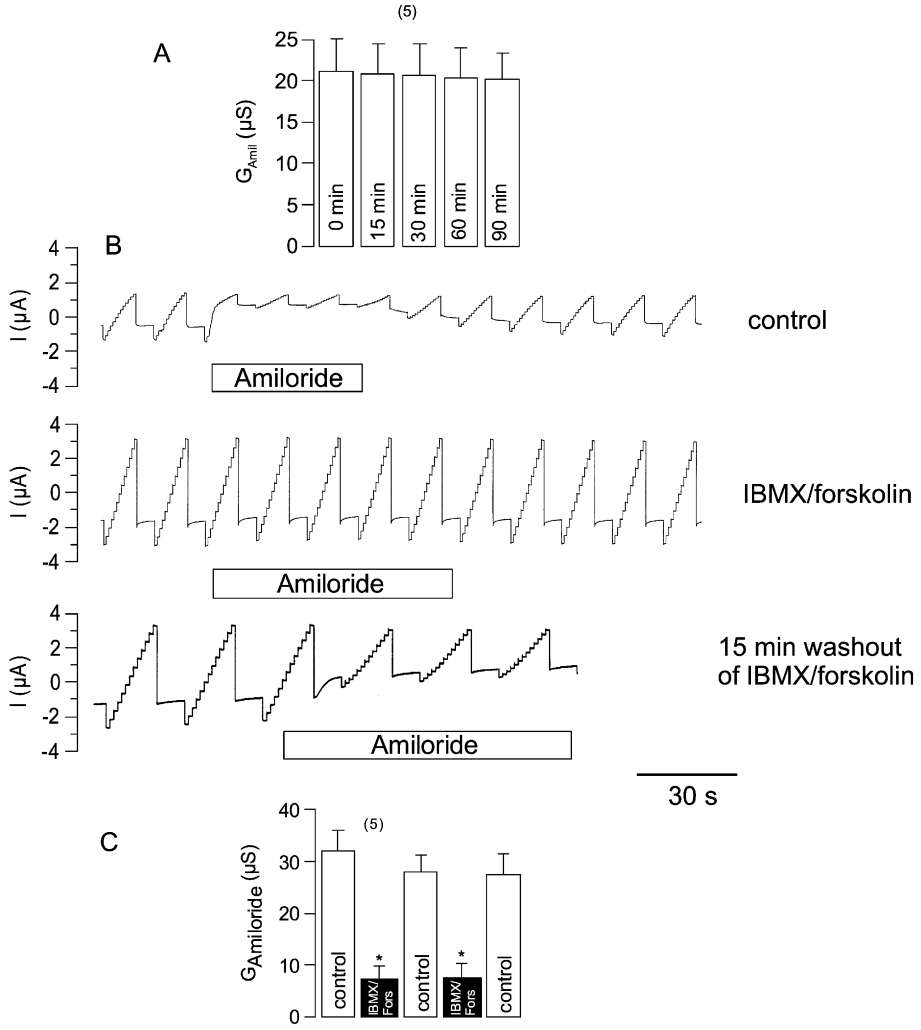


Fig. 1. Expression of ENaC and CFTR in *Xenopus* oocytes. (A) Continuous recording of amiloride-sensitive whole cell conductance ( $G_{Amiloride}$) over 90 min does not show any run down of $G_{Amiloride}$. (B) Original recordings of the whole cell currents obtained in a CFTR/ENaC expressing oocyte. Activation of CFTR by stimulation with IBMX (1 mmol/l) and forskolin (2 µmol/l) inhibits the amiloride-sensitive whole cell current. The inhibition shows a partial recovery of $G_{Amiloride}$ 15 min after omission of IBMX/forskolin. (C) $G_{Amiloride}$ can be inhibited repetitively by activating and deactivating CFTR. * indicate significant difference from control (paired *t*-test). (Number of experiments).

sanne, Switzerland), human CFTR, mouse nucleoside diphosphate kinase A (NDPK) and rat $P2Y_2$ receptor were linearized in pBluescript or pTLN [12] with *Not*I or *Mlu*I, and in vitro transcribed using T7, T3 or SP6 promotor and polymerase (Message Machine, Ambion, USA). Isolation and microinjection of oocytes have been described in a previous report [9]. In brief, after isolation from adult *Xenopus laevis* female frogs (*Xenopus* express, South Africa), oocytes were dispersed and defolliculated by a 0.8-h 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).

### 2.2. 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. Two to four days after injection, oocytes were impaled with two electrodes (Clark Instruments), which had a resistance of < 1 MΩ when filled with 2.7 mol/l KCl. Two bath electrodes were used, which had 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 to + 30 mV, in steps of 10 mV, each 1 s.

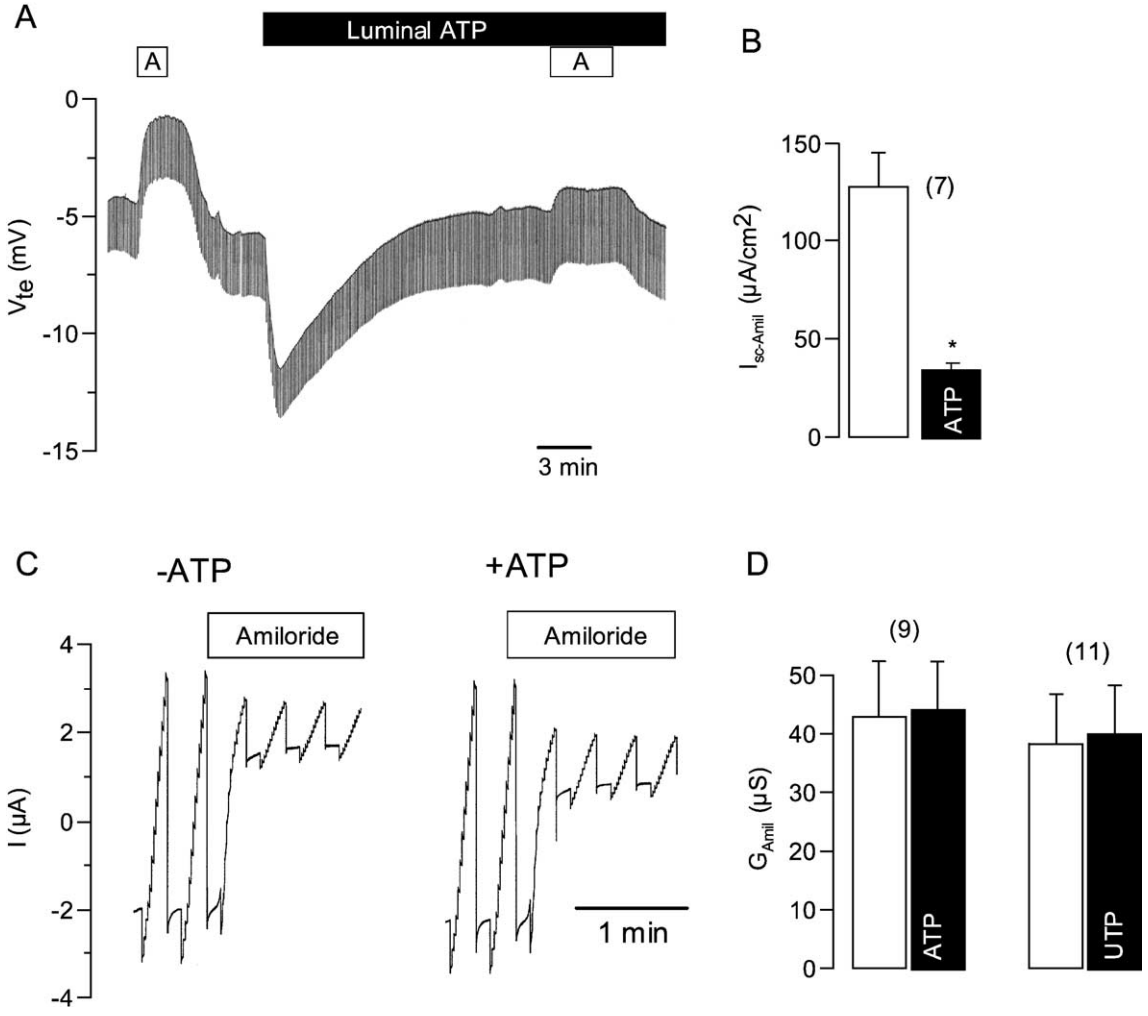


Fig. 2. Effects of stimulation of luminal purinergic receptors by ATP or UTP on ion transport in mouse trachea and *Xenopus* oocytes expressing ENaC. (A) Continuous recording of the transepithelial voltage ($V_{te}$) in mouse trachea. ATP (100 μmol/l) induced a transient voltage deflection and inhibited amiloride (A) sensitive transport. (B) Summary of the amiloride-sensitive short circuit currents before and after stimulation with ATP. (C) Whole cell currents measured in *Xenopus* oocytes expressing the epithelial $Na^+$ channel ENaC and effects of amiloride (10 μmol/l) in the absence or presence of ATP (100 μmol/l). (D) Summary of the amiloride-sensitive whole cell conductances ( $G_{Amil}$) measured in ENaC expressing oocytes. Stimulation by ATP or UTP (both 100 μM) has no impact on $G_{Amil}$. (Number of experiments).

Current data were filtered at 50 Hz. Data were collected continuously (PowerLab, AD-Instruments, Australia) and were analyzed by using the programs chart and scope (PowerLab, AD-Instruments). Conductances were calculated according to Ohm's law and amiloride-sensitive conductances ($G_{Amil}$) is 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).

### 2.3. Ussing chamber experiments

Tracheas were taken from mice (Quackenbush, animal facility of the University of Queensland) after sacrificing the animal by cervical dislocation, and opened by a longitudinal cut after connective tissues were removed. Mouse distal colon was removed from the animal and the mucosa was separated mechanically from the submucosal tissue. Small superficial mucosal biopsies were obtained from human rectum of 11 individuals (Department of Pediatrics, University of Freiburg, Germany). The study has been approved by the ethical committee of the University of Freiburg, Germany. 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

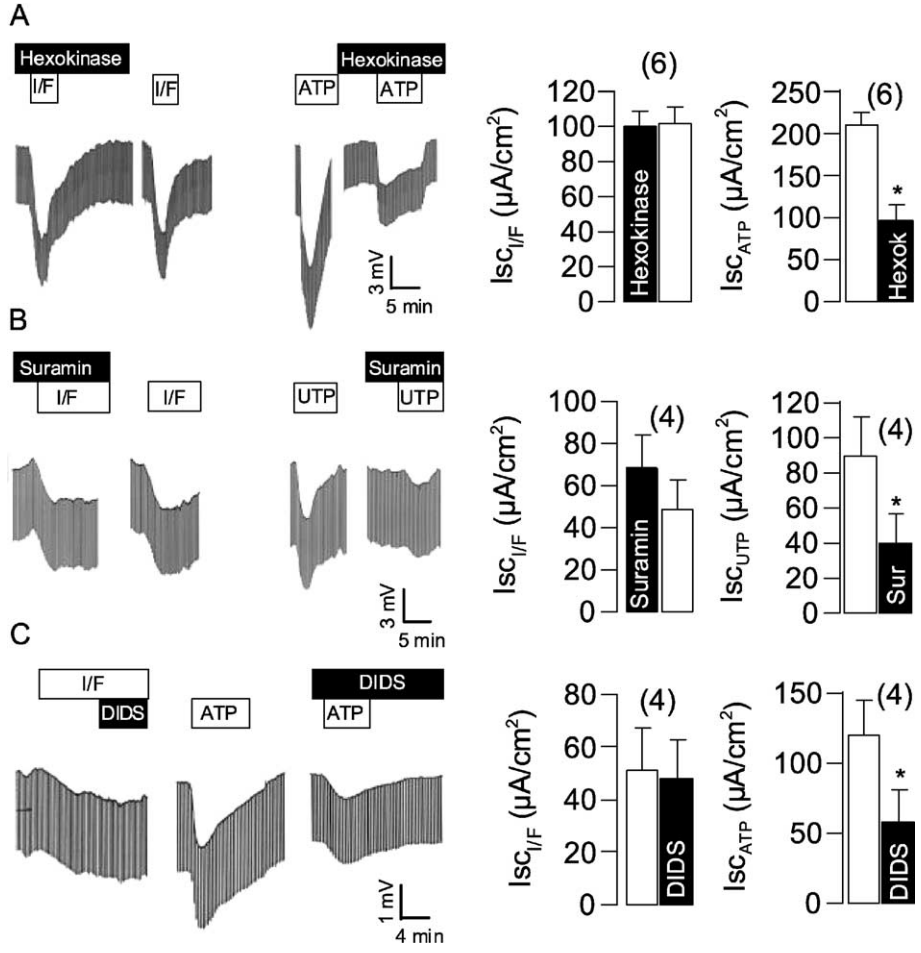


Fig. 3. Effects of stimulation by IBMX (100 μmol/l)/forskolin (2 μmol/l) (I/F) and ATP or UTP (both 100 μmol/l) on transepithelial voltages and summary of short circuit currents measured in mouse trachea. (A) Incubation with hexokinase (5 U/ml) and glucose (15 mM) attenuates ATP-activated transport but has no effect on I/F induced secretion. (B) The P2Y blocker suramin (100 μmol/l) inhibits UTP-activated transport but has no effect on I/F-induced secretion. (C) The $Cl^-$ channel blocker DIDS inhibits ATP-activated transport but has no effect on I/F-induced secretion. * indicate significant difference from control (paired *t*-test). (Number of experiments).

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 Ohms 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 3–4 h.

### 2.4. Materials and statistical analysis

All used compounds were of highest available grade of purity. ATP, UTP, suramin, carbachol, 3-isobutyl-1-methyl-xanthine (IBMX), membrane-permeable $N^2$,2′-*O*-dibutyryl-

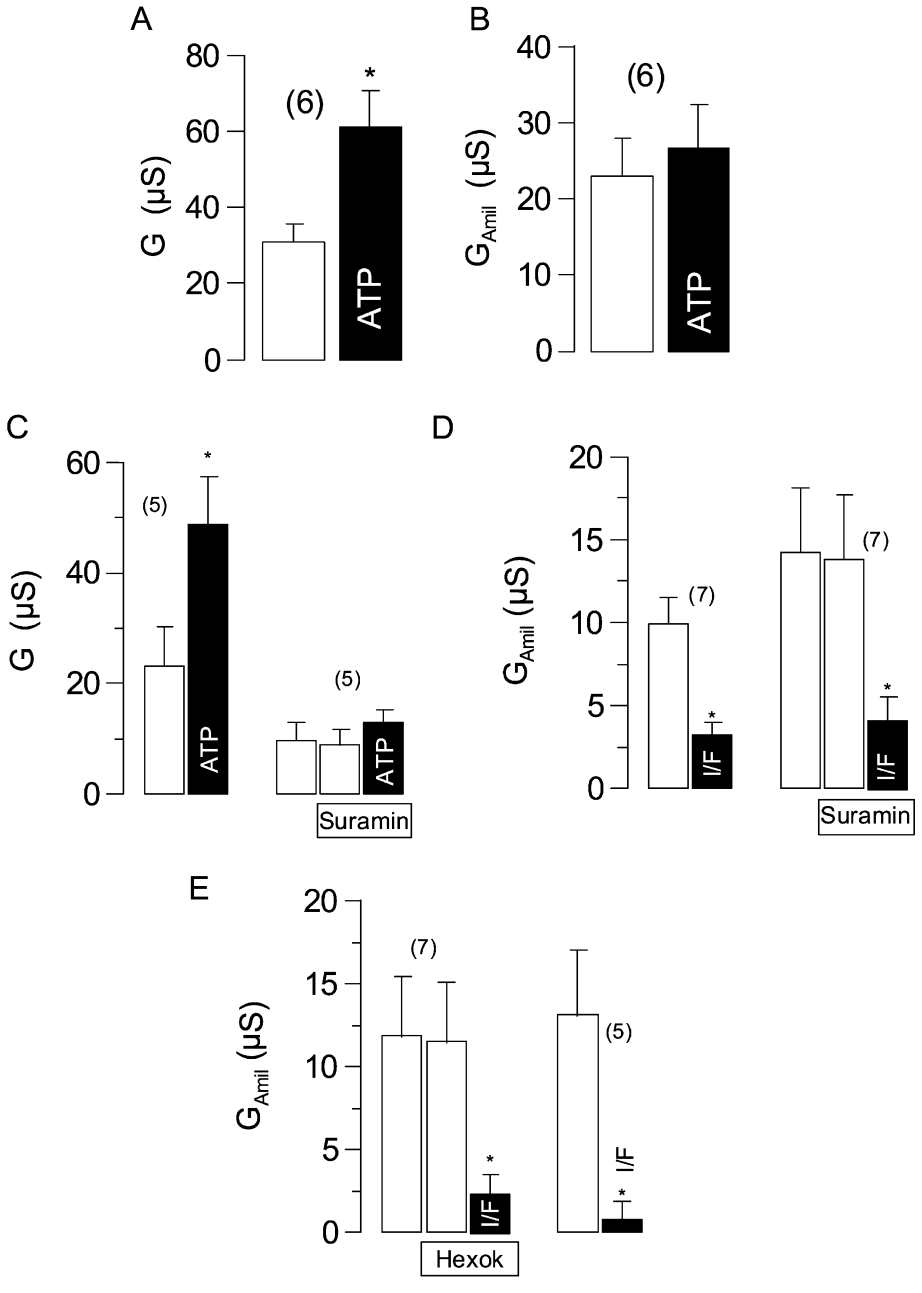


Fig. 4. Summary of whole cell conductances measured in *Xenopus* oocytes coexpressing ENaC and $P2Y_2$ receptors. A whole cell $Cl^-$ conductance is activated by stimulation with ATP (100 μmol/l) (A), which does not affect amiloride-sensitive $Na^+$ conductance ($G_{Amil}$) (B). (C–E) Summary of whole cell conductances measured in oocytes coexpressing ENaC, $P2Y_2$ receptors and CFTR. A whole cell $Cl^-$ conductance is activated by stimulation with ATP (100 μmol/l). Suramin itself has no effects on basal ion conductance (C) or amiloride sensitive conductance (D). However, the effect of ATP is completely suppressed by suramin. Activation of CFTR by IBMX (1 mmol/l) and forskolin (2 μmol/l) (I/F) inhibits $G_{Amil}$ in the absence or presence of suramin (D) or hexokinase (5 U/ml) and glucose (15 mmol/l) (E). * indicate significant difference from control. (Number of experiments).

guanosine 3′,5′-cyclic monophosphate (cGMP), forskolin, amiloride, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), hexokinase and naringenin were all from Sigma (Australia). The peptide-inhibitor of protein kinase G (PKG inhibitor) H-Arg-Lys-Arg-Ala-Arg-Lys-glu-OH was from Calbiochem (Australia). Student's *t*-test *P* values $<0.05$ were accepted to indicate statistical significance. (Number of experiments).

## 3. Results

### 3.1. Inhibition of ENaC by CFTR and purinergic stimulation

When both CFTR and ENaCs were coexpressed in *Xenopus* oocytes, an amiloride-sensitive whole cell conductance was detected, which did not show a rundown during 90-min observation (Fig. 1A). The amiloride-sensitive whole cell current was inhibited upon stimulation of CFTR by IBMX (1 mmol/l) and forskolin (2 μmol/l). The inhibition was partially reversible already after 15-min washout of IBMX and forskolin (Fig. 1B). A complete recovery of the amiloride-sensitive conductance was obtained after omitting IBMX and forskolin for 30 min and deactivation of CFTR. Subsequent restimulation of CFTR again inhibited $G_{Amiloride}$ (Fig. 1C). Thus, the data indicate a reversible and reproducible inhibition of ENaC by CFTR in *Xenopus* oocytes, which cannot be explained by a rundown of ENaC channel activity. A similar reversible inhibition of amiloride-sensitive transport by CFTR has been demonstrated in native airway and colonic epithelia [24,26] and is also shown in Fig. 5 of this paper, emphasizing on the physiological significance of this finding.

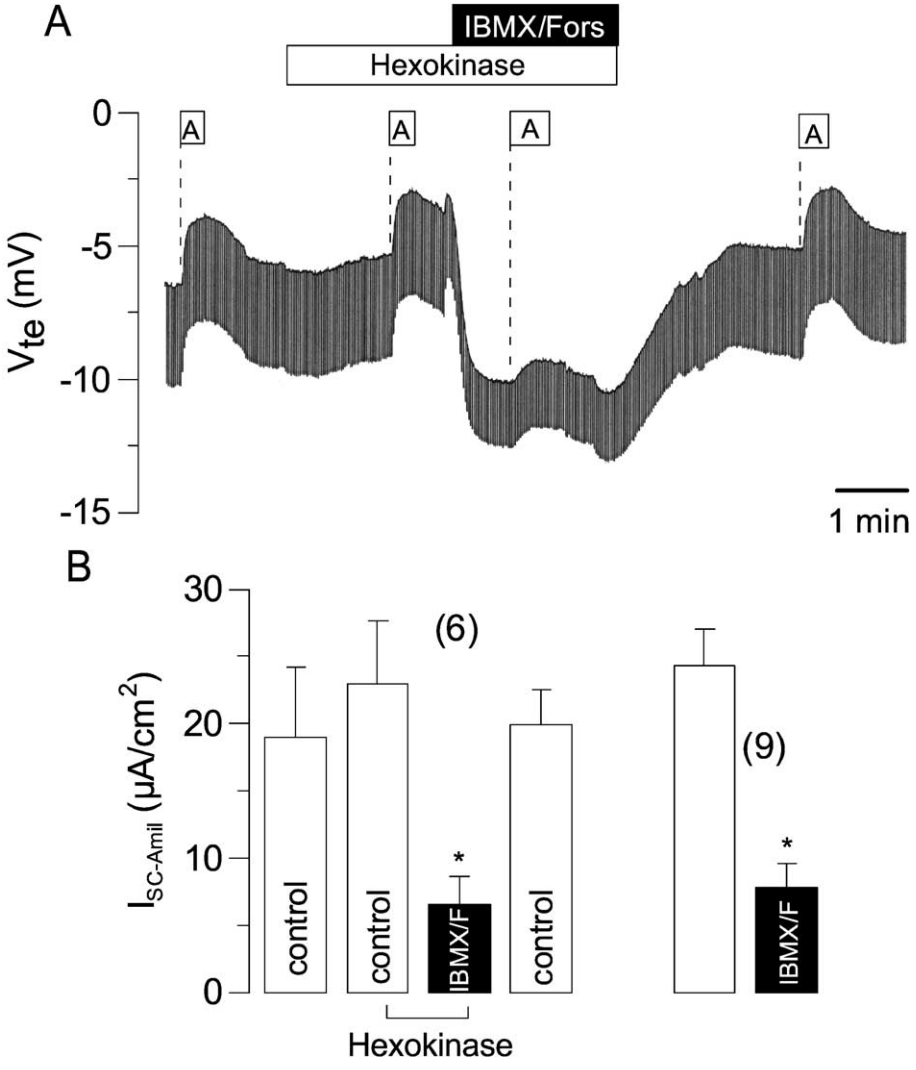


Fig. 5. (A) Continuous recording of the transepithelial voltage ($V_{te}$) in mouse colon and effects of amiloride on $V_{te}$ in the presence or absence of IBMX/forskolin and hexokinase/glucose. (B) Summary of the amiloride-sensitive short circuit currents ($I_{sc\text{-}Amil}$) obtained before and after stimulation of CFTR. $I_{sc\text{-}Amil}$ is inhibited by CFTR, even in the presence of hexokinase/glucose. Inhibition in the presence of hexokinase/glucose is not different to inhibition of $I_{sc\text{-}Amil}$ in the absence of hexokinase/glucose. * indicate significant difference from control (paired *t*-test). (Number of experiments).

Similar to CFTR, also purinergic stimulation inhibits amiloride-sensitive $Na^+$ transport in the airway epithelium. When stimulated with 100 μmol/l ATP from the apical side, mouse tracheas responded with a large increase in the lumen negative transepithelial voltage ($V_{te}$), indicating transient activation of $Cl^-$ secretion

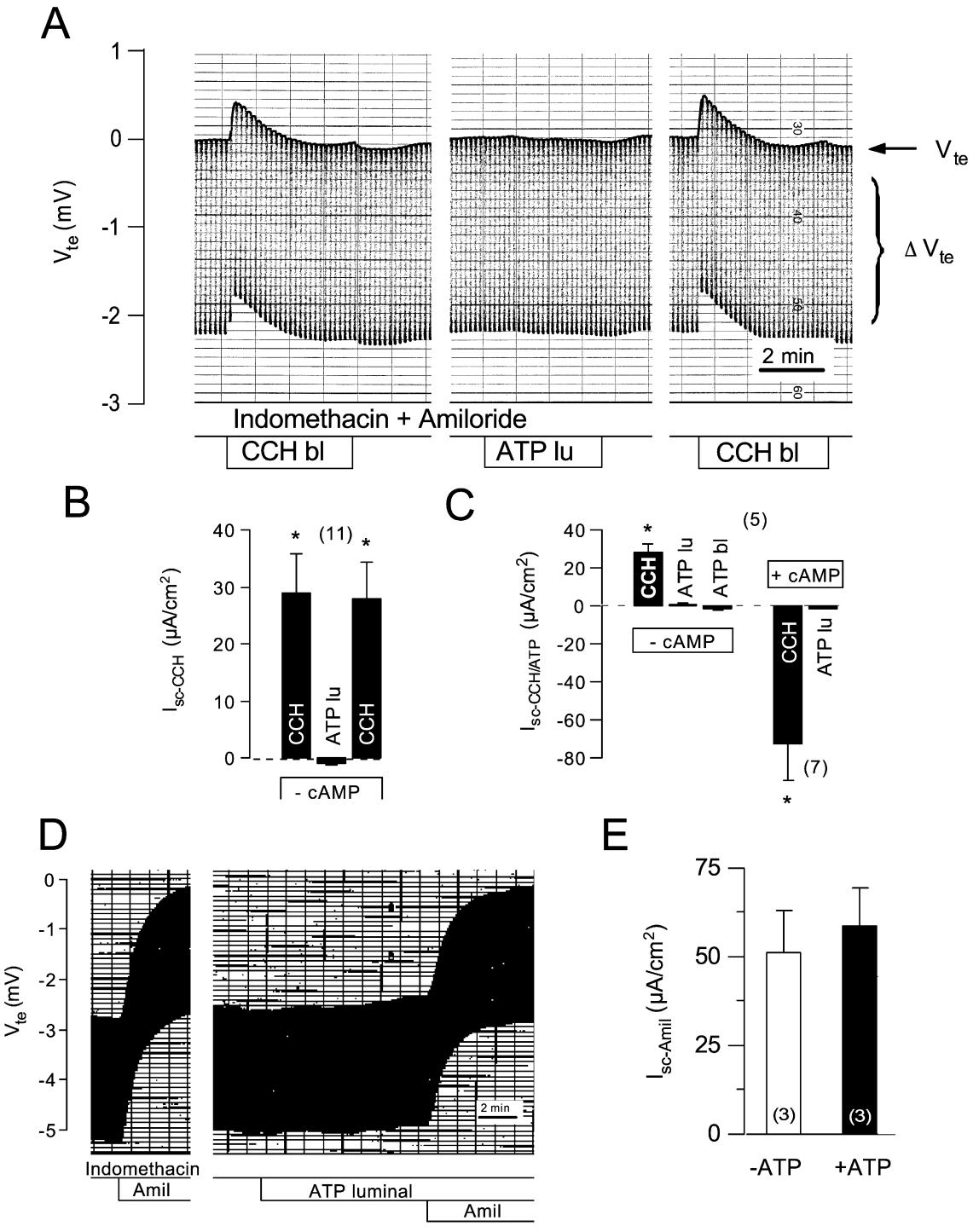


Fig. 6. Transepithelial voltages measured in human colonic biopsies. (A) Continuous recording of the transepithelial voltage ($V_{te}$) in human colonic epithelia and effects of stimulation by basolateral carbachol (CCH; 100 μmol/l) and luminal UTP (100 μmol/l). Experiments were performed in the continuous presence of indomethacin and amiloride (both 10 μmol/l). $\Delta V_{te}$ = voltage deflection induced by pulsed current injection. (B) Summary of short circuit currents induced by basolateral CCH and luminal ATP in the absence of stimulation by IBMX/forskolin ( − cAMP). (C) Summary of short circuit currents induced by basolateral CCH and basolateral or luminal ATP in the absence ( − cAMP) or after stimulation by IBMX/forskolin ( + cAMP). No effect of ATP on short circuit currents could be detected. (D,E) Missing effect of stimulation by luminal ATP on $I_{sc\text{-}Amil}$. * indicate significant difference from control (paired *t*-test). (Number of experiments).

(Fig. 2A). In parallel, the effect of amiloride on $V_{te}$ and amiloride-sensitive short circuit currents were largely inhibited (Fig. 2A,B). The inhibitory effects of purinergic stimulation on amiloride-sensitive transport were reversible within 90-min washout of ATP (data not shown). We further examined the effects of purinergic stimulation on ENaC in *Xenopus* oocytes. When applied to ENaC expressing oocytes, we did not detect an inhibitory effect of ATP or UTP on amiloride-sensitive conductance, i.e. whole cell currents inhibited by amiloride were similar in the absence or presence of ATP or UTP (Fig. 2C,D). These experiments exclude the possibility of a direct inhibitory effect of purinergic agonists on ENaC and indicate the requirement of additional proteins necessary for inhibition of ENaC.

### *3.2. Inhibition of ENaC by CFTR via release of ATP or UTP?*

We examined the possibility that CFTR is controlling the release of purinergic agonists to the luminal membrane of the airway epithelium, where they would bind to P2Y receptors, activate a $Ca^{2+}$-dependent $Cl^-$ conductance and inhibit amiloride-sensitive $Na^+$ channels. To that end, tracheas were stimulated with IBMX and forskolin in the presence or absence of hexokinase (5 U/ml) and glucose (15 mmol/l). Hexokinase and glucose were applied in order to metabolize nucleotides, eventually secreted to the luminal side of the epithelium during activation of CFTR. In case of a substantial CFTR-controlled release of nucleotides to the luminal side, we would expect a parallel activation of cAMP and $Ca^{2+}$-

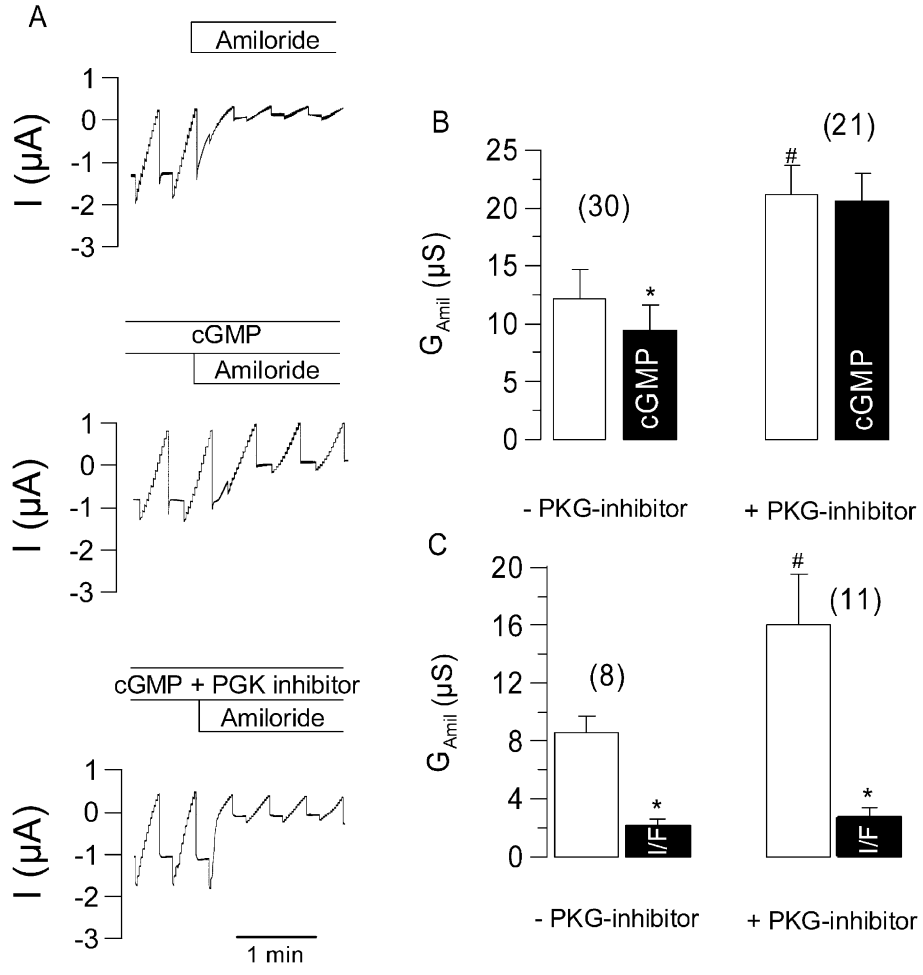


Fig. 7. Effects of cGMP on ENaC expressed in *Xenopus* oocytes. (A) Continuous recording of the whole cell currents measured in *Xenopus* oocytes expressing the epithelial $Na^+$ channel ENaC, and effects of amiloride (10 μmol/l) (upper trace). Incubation with 1 mmol/l of membrane-permeable cGMP inhibits ENaC (middle trace). The inhibitory effect of cGMP on ENaC is suppressed by an inhibitor of protein kinase G (lower trace). (B) Summary of the effects of membrane permeable cGMP on amiloride-sensitive whole cell conductance ($G_{Amil}$) in the absence or presence of the PKG inhibitor. Note that cGMP has a slight but significant inhibitory effect on $G_{Amil}$ only in the absence of PKG inhibitor. The PKG inhibitor enhanced $G_{Amil}$. (C) Downregulation of $G_{Amil}$ by activation of CFTR with IBMX (1 mmol/l) and forskolin (2 μmol/l) (I/F) takes place in the absence or presence of the PKG inhibitor. # indicate significantly enhanced $G_{Amil}$ in the presence of PKG inhibitor (unpaired *t*-test). * indicate significant difference from control (paired *t*-test). (Number of experiments).

activated $Cl^-$ conductance and thus an attenuated secretion in the presence of hexokinase and glucose. Although ATP-induced ion transport was inhibited, IBMX/forskolin (I/F) activated transport was identical in the presence or absence of hexokinase/glucose (Fig. 3A). Similarly, the purinergic inhibitor suramin (200 μmol/l) attenuated UTP-induced transport but had no effect on I/F activated secretion (Fig. 3B). Finally, the blocker of $Ca^{2+}$-activated $Cl^-$ channels DIDS attenuated ATP-induced transport, but was without any effect on I/F induced secretion (Fig. 3C). Thus, the present experiments do not deliver any evidence for CFTR-mediated nucleotide secretion and activation of $Ca^{2+}$-dependent $Cl^-$ channels. We therefore suggest that CFTR-mediated inhibition of ENaC is not due to release of ATP/UTP to the luminal side of the epithelium.

We further examined a possible contribution of nucleotide release to CFTR-mediated inhibition of ENaC in *Xenopus* oocytes. To that end, we coexpressed purinergic $P2Y_2$ receptors together with ENaC in *Xenopus* oocytes. As shown in Fig. 4A, ATP activates a whole cell conductance in $P2Y_2$/ENaC expressing oocytes. However, no inhibition of the amiloride-sensitive conductance was observed during stimulation by ATP. In another series of experiments, $P2Y_2$ receptors and ENaC were coexpressed with CFTR. In this series of experiments, the transient whole cell conductance activated by ATP (100 μmol/l) was completely suppressed by suramin (Fig. 4C). Suramin, however, did not suppress inhibition of $G_{Amil}$ due to activation of CFTR, which again argues against a CFTR-mediated ATP/UTP release and a role for inhibition of ENaC. Thus, inhibition of $G_{Amil}$ was similar in the absence or presence of suramin (Fig. 4D). Along this line, hexokinase and glucose did not suppress I/F-induced inhibition of $G_{Amil}$ (Fig. 4E).

The possibility of ATP/UTP release by CFTR in the intact epithelium was further ruled out. Previous studies have shown that CFTR inhibits amiloride-sensitive currents in native human and mouse colonic epithelia [26,42]. In the present study, we examined whether inhibition of amiloride-sensitive $Na^+$ transport by CFTR in mouse colon may involve secretion of nucleotides. Amiloride-sensitive transport was measured before and after stimulation of CFTR by IBMX (100 μmol/l) and forskolin (10 μmol/l) and in the presence of hexokinase (5 mmol/l) and glucose (15 mmol/l). As shown in Fig. 5, amiloride-induced voltage deflections were attenuated after stimulation of CFTR and amiloride-sensitive transport ($I_{sc\text{-}Amil}$) was reduced even in the presence of hexokinase and glucose. Additional experiments were performed in human colonic biopsies. A previous study has demonstrated inhibition of $I_{sc\text{-}Amil}$ by CFTR in the human colon [26]. Since little is known about the effects of purinergic stimulation in this tissue, we examined the impact of luminal and basolateral ATP (100 μmol/l) on ion transport. The experiments were performed in the presence of amiloride, after deactivating luminal CFTR $Cl^-$ channels by inhibiting prostaglandin synthesis with indomethacin [28], or after stimulation of CFTR by IBMX and forskolin. After inactivation of CFTR, increase of intracellular $Ca^{2+}$ by stimulation of basolateral muscarinic M3 receptors with carbachol (CCH; μmol/l) activates a luminal $K^+$ secretion. Similar has been reported previously [25,28,41]. In contrast to CCH, luminal ATP had no effect on transepithelial voltage and ion transport in the human colon (Fig. 6A,B). Moreover, neither luminal nor basolateral ATP showed effects after stimulation of CFTR (+cAMP), while stimulation with CCH induced a $Cl^-$ secretion. Finally, amiloride-sensitive transport was not inhibited by stimulation with luminal ATP (Fig. 6D,E). Taken together, these results suggest that in contrast to the murine colon [18], the human colonic epithelium does not express purinergic receptors. Thus, the inhibitory effects of CFTR on ENaC in the human colon cannot be explained by release of ATP or UTP.

### 3.3. cGMP and NDPK do not participate in the inhibition of ENaC by CFTR

Other ATP-dependent processes may play a role for the inhibition of ENaC. The ATP and ion regulated dependent NDPK has been shown to be expressed in airway epithelial cells, where it may interfere with the ion transport

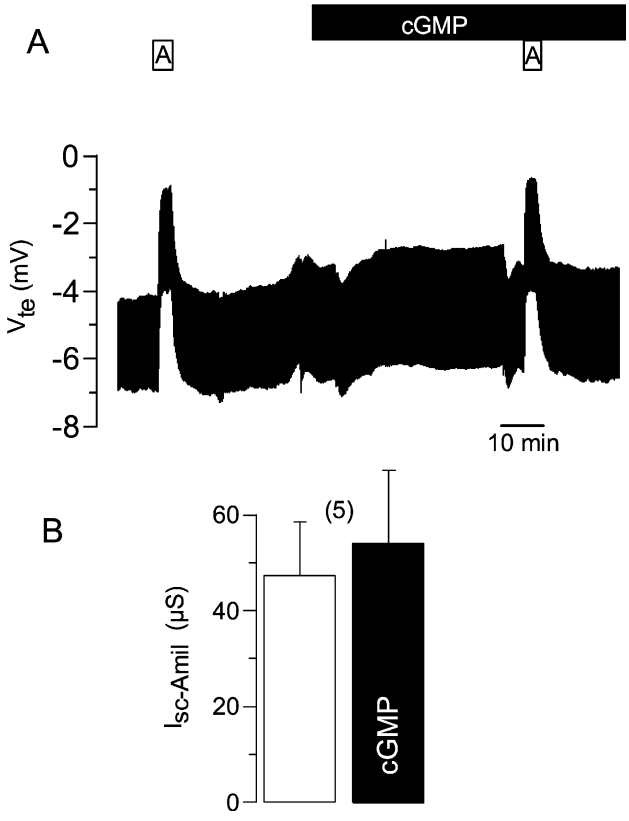


Fig. 8. (A) Continuous recording of the transepithelial voltage ($V_{te}$) in mouse trachea and effects of amiloride on $V_{te}$ in the presence or absence of membrane permeable cGMP. (B) Summary of the amiloride-sensitive short circuit currents ($I_{sc\text{-}Amil}$) obtained before and application of membrane permeable cGMP. (Number of experiments).

[29,31,39]. Moreover, it has been shown recently that NDPK binds to and is regulated by adenosine monophosphate dependent kinase (AMPK), which is a CFTR-associated protein defective in CF [40]. Upon activation of NDPK, ATP is metabolized to UTP or GTP, which could contribute to the formation of cGMP and inhibit ENaC [5]. This model is further supported by the fact that both activation of NDPK and inhibition of ENaC by CFTR are $Cl^-$-dependent processes [14,31]. We examined the effects of cGMP on amiloride-sensitive $Na^+$ currents in oocytes. As shown in Fig. 7, the amiloride-sensitive $Na^+$ current is inhibited slightly but significantly in the presence of 1 mmol/l membrane-permeable cGMP (Fig. 7A,B). Inhibition of ENaC by cGMP was abolished by injecting an inhibitor of PKG. Interestingly, amiloride-sensitive whole cell conductances were enhanced in oocytes injected with PKG inhibitor, which may suggest a basic inhibition of ENaC by PKG in *Xenopus* oocytes. However, an inhibitory effect of cGMP on amiloride-sensitive transport could not be detected in mouse trachea (Fig. 8). Moreover, the downregulation of ENaC by CFTR was undisturbed by the presence of the PKG inhibitor (Fig. 7C). In addition, the inhibition of ENaC by CFTR was examined in oocytes, which had been injected with naringenin (final concentration 500 μmol/l), a blocker of NDPK, or was demonstrated in oocytes that coexpressed NDPK (Fig. 9A,B). Neither naringenin nor NDPK interfered with the downregulation of ENaC by CFTR. In further experiments, we tested the effects of fisetin and naringenin on mouse airways, but did not find any significant effects on $Cl^-$ secretion or $Na^+$ absorption (data not shown). Taken together, the present results do not support a role of either CFTR-mediated release of ATP, or ATP-dependent NDPK for the inhibition of ENaC by CFTR.

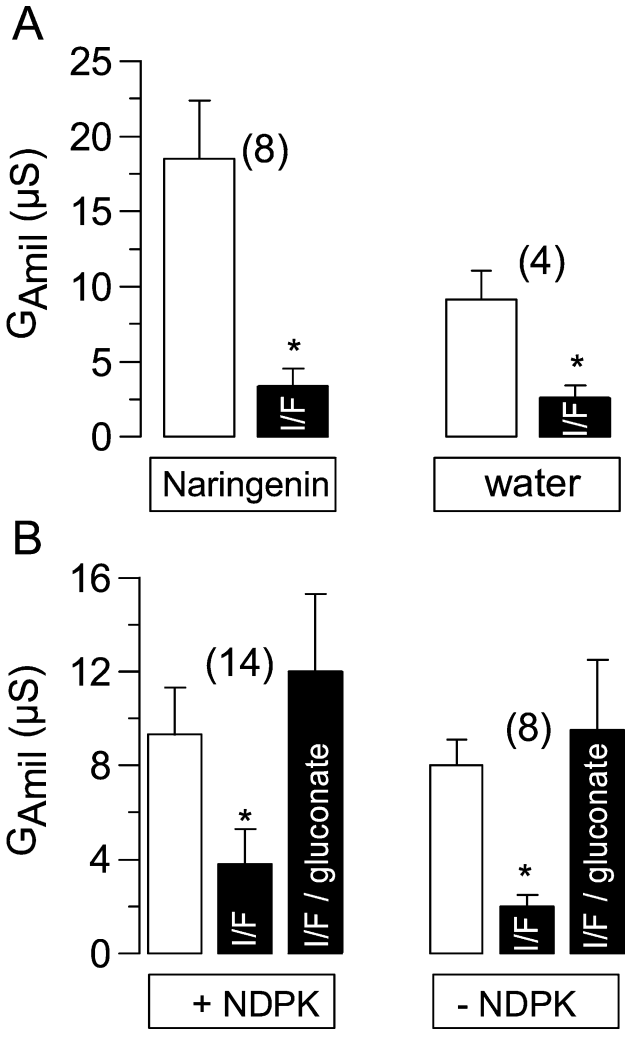


Fig. 9. Summary of amiloride-sensitive whole cell conductances ($G_{Amil}$) measured in *Xenopus* oocytes coexpressing ENaC and CFTR. (A) Oocytes were injected with the inhibitor of nucleoside diphosphate kinase, naringenin (final oocyte concentration 50 μmol/l), or water. Downregulation of $G_{Amil}$ by activation of CFTR with IBMX (1 mmol/l) and forskolin (2 μmol/l) (I/F) was not suppressed by naringenin. (B) Additional coexpression of NDPK did not affect downregulation of $G_{Amil}$ by activation of CFTR with IBMX and forskolin. Inhibition of $G_{Amil}$ was reversible upon replacement of extracellular $Cl^-$ by gluconate. * indicate significant difference from control (paired *t*-test). (Number of experiments).

## 4. Discussion

### *4.1. ENaC is inhibited by CFTR and by purinergic stimulation*

Previous results and our present results indicate that amiloride-sensitive $Na^+$ transport is inhibited by both CFTR and purinergic stimulation in native epithelial tissues [2,24,26,27]. When coexpressed in *Xenopus* oocytes, ENaC is inhibited during activation of CFTR. As shown in this study, downregulation of ENaC by CFTR is reproducible in the same oocyte and is not due to ENaC channel rundown. However, in contrast to inhibition by CFTR, purinergic stimulation does not inhibit ENaC in *Xenopus* oocytes. Even in oocytes expressing additional purinergic $P2Y_2$ receptors, thus clearly demonstrating ATP/UTP-activated whole cell $Cl^-$ currents, inhibition of ENaC by ATP or UTP was not observed. Obviously, in *Xenopus* oocytes, a regulatory element is missing, necessary for inhibition of ENaC. As another explanation, activation of $P2Y_2$ receptors with the subsequent signaling pathway might be too transient in oocytes, especially when compared to the airway epithelium. As shown previously, the downregulation of amiloride-sensitive short circuit currents in mouse trachea requires 5–10 min of continuous stimulation with ATP or UTP and a longer lasting activation of $Ca^{2+}$-activated $Cl^-$ currents [20]. In the present study, we did not directly measure ATP release by either epithelial cells or oocytes. Instead, we performed a functional analysis of the effects of purinergic stimulation in various tissues and compared the effects of ATP/UTP on $Cl^-$ conductance and amiloride-sensitive $Na^+$ absorption. Although any conclusions regard-

ing ATP/UTP release are indirect, the data obtained here do not supply any evidence for CFTR-dependent nucleotide release and do not indicate a role for ATP/UTP in inhibition of ENaC by CFTR in *Xenopus* oocytes.

### *4.2. No evidence for release of ATP and activation of CaCC during stimulation of CFTR*

We sought for functional evidence of ATP release, not by measuring ATP release directly but by detecting activation of CaCC during stimulation of CFTR in mouse trachea. Although a recent study demonstrated very low expression of CFTR in mouse trachea [34], our experiments show activation of $Cl^-$ secretion by CFTR, as indicated by cAMP-induced negative voltage deflection. Activation of short circuit currents by cAMP, however, was not affected by hexokinase, a blocker of purinergic receptors (suramin), or $Ca^{2+}$-activated $Cl^-$ channels (DIDS). This argues against any contribution of purinergic stimulation to CFTR-mediated secretion in mouse trachea. Therefore, our data are in agreement with previously published reports, which deny a role of CFTR in luminal ATP release in the airway epithelium [7,22,23,33,38,43]. The same holds true for the mouse intestinal epithelium. Here, cAMP (IBMX and forskolin) activated secretion and inhibition of amiloride-sensitive transport was not affected by hexokinase treatment. Finally, CFTR-dependent inhibition of $Na^+$ absorption described for the human intestinal epithelium [26] is unrelated to ATP/UTP release since no evidence was found in the present study for expression of purinergic receptors in the human large intestine and no effects of ATP on $I_{sc\text{-}Amil}$ could be detected. Taken together, the present study does not support the idea of an autocrine release of ATP by CFTR and inhibition of amiloride-sensitive currents via this mechanism [36].

### *4.3. Inhibition of ENaC by CFTR is not due to NDPK function or cGMP-dependent inhibition*

Regulation of ENaC by CFTR has been studied extensively and recent results indicated that ENaC might be inhibited through the CFTR $Cl^-$ current and a change in the intracellular $Cl^-$ concentration [14]. This asks for the contribution of additional $Cl^-$-sensitive proteins to the inhibition of ENaC. A $Cl^-$-dependent mechanism has been reported for the fetal rat alveolar epithelium and the feedback inhibition of ENaC in salivary duct cells [4,13,30]. In salivary epithelial cells, αGo and αGi2 proteins mediate the inhibition of ENaC via increase in intracellular $Na^+$ and $Cl^-$, respectively [3,4,13,30]. However, a contribution of these G protein subunits to the $Na^+$ feedback or CFTR-dependent regulation of ENaC in oocytes has not been found [10,17]. In the present study, we examined the possible role of NDPK, another $Cl^-$-and cation-dependent enzyme. NDPK is expressed in the airway epithelium and is regulated by AMPK, which has been shown to be a CFTR-associated protein being defective in cystic fibrosis [31,39,40]. NDPK has been suggested to play a role in the control of ion channels in the airway epithelium [31,39] and thus could be the protein that is in charge of CFTR-mediated inhibition of ENaC. The data shown here, however, do not support the idea of NDPK, being the $Cl^-$-sensitive protein in charge of inhibition of ENaC in airways or oocytes. Moreover, although cGMP- and/or cGMP-dependent PKG may inhibit ENaCs in some cell types such as *Xenopus* oocytes or murine nasal epithelium, this regulatory loop does not affect the control of ENaC by CFTR [5]. Taken together, it is unlikely that a release of ATP or UTP contributes to the inhibition of ENaC by CFTR. This, however, should have been expected if CFTR shows a significant nucleotide transport. Other ATP-dependent signaling pathways such as via NDPK or cGMP are also unlikely to play a role. Since $Cl^-$ was found to be crucial for the inhibition of ENaC, current studies examine if $Cl^-$ ions directly inhibit $Na^+$ transport through ENaC. Preliminary results suggest that N or C termini of ENaC might not be required for the inhibition by CFTR, which suggests a possible direct inhibition by $Cl^-$ ions [42].

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## References

[1] G.M. Braunstein, R.M. Roman, J.P. Clancy, B.A. Kudlow, A.L. Taylor, V.G. Shylonsky, B. Jovov, K. Peter, T. Jilling, I.I. Ismailov, D.J. Benos, L.M. Schwiebert, J.G. Fitz, E.M. Schwiebert, Cystic fibrosis transmembrane conductance regulator facilitates ATP release by stimulating a separate ATP release channel for autocrine control of cell volume regulation, J. Biol. Chem. 276 (2001) 6621–6630.
[2] D.C. Devor, J.M. Pilewski, UTP inhibits $Na^+$ absorption in wild-type and DeltaF50B CFTR-expressing human bronchial epithelia, Am. J. Physiol. 276 (1999) C827–C837.
[3] A. Dinudom, K.F. Harvey, P. Komwatana, J.A. Young, S. Kumar, D.I. Cook, Nedd4 mediates control of an epithelial $Na^+$ channel in salivary duct cells by cytosolic $Na^+$, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7169–7173.
[4] A. Dinudom, J.A. Young, D.I. Cook, $Na^+$ and $Cl^-$ conductances are controlled by cytosolic $Cl^-$ concentration in the intralobular duct cells of mouse mandibular glands, J. Membr. Biol. 135 (1993) 289–295.
[5] H.L. Elmer, K.G. Brady, M.L. Drumm, T.J. Kelley, Nitric oxide-mediated regulation of transepithelial sodium and chloride transport in murine nasal epithelium, Am. J. Physiol. 276 (1999) L466–L473.
[6] H. Garty, L.G. Palmer, Epithelial sodium channels: function, structure and regulation, Physiol. Rev. 77 (1997) 359–396.
[7] R. Grygorczyk, J.W. Hanrahan, CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli, Am. J. Physiol. 272 (1997) C1058–C1066.
[8] R. Grygorczyk, J.A. Tabcharani, J.W. Hanrahan, CFTR channels ex-

pressed in CHO cells do not have detectable ATP conductance, J. Membr. Biol. 151 (1996) 139–148.
[9] A. Hipper, M. Mall, R. Greger, K. Kunzelmann, Mutations in the putative pore forming domain of CFTR do not change anion selectivity of the cAMP activated $Cl^-$ conductance, FEBS Lett. 374 (1995) 312–316.
[10] M. Hübner, R. Schreiber, A. Boucherot, A. Sanchez-Perez, P. Poronnik, D.I. Cook, K. Kunzelmann, Feedback inhibition of epithelial $Na^+$ channels in *Xenopus* oocytes does not require Go- or Gi2-proteins, FEBS Lett. 459 (1999) 443–447.
[11] B. Jovov, I.I. Ismailov, D.J. Benos, Cystic fibrosis transmembrane conductance regulator is required for protein kinase A activation of an outwardly rectified anion channel purified from bovine tracheal epithelia, J. Biol. Chem. 270 (1995) 1521–1528.
[12] S. Kieferle, P. Fong, M. Bens, A. Vandewalle, T.J. Jentsch, Two highly homologous members of the CIC chloride channel family in both rat and human kidney, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6943–6947.
[13] P. Komwatana, A. Dinudom, J.A. Young, D.I. Cook, Cytosolic $Na^+$ controls an epithelial $Na^+$ channel via Go guanine nucleotide-binding regulatory protein, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 8107–8111.
[14] J. König, R. Schreiber, T. Voelcker, M. Mall, K. Kunzelmann, CFTR inhibits ENaC through an increase in the intracellular CI-concentration, EMBO Rep. 2 (2001) 1–5.
[15] K. Kunzelmann, The cystic fibrosis transmembrane conductance regulator and its function in epithelial transport, Rev. Physiol., Biochem. Pharmacol. 137 (1999) 1–70.
[16] K. Kunzelmann, CFTR: interacting with everything? News Physiol. Sci. 17 (2001) 167–170.
[17] K. Kunzelmann, A. Boucherot, Mechanism of the inhibition of epithelial $Na^+$ channels by CFTR and purinergic stimulation, Kidney Int. 60 (2001) 455–461.
[18] K. Kunzelmann, M. Mall, Electrolyte transport in the colon: mechanisms and implications for disease, Physiol. Rev. 82 (2001) 245–289.
[19] K. Kunzelmann, R. Schreiber, CFTR, a regulator of channels, J. Membr. Biol. 168 (1999) 1–8.
[20] K. Kunzelmann, R. Schreiber, D.I. Cook, Mechanisms for inhibition of amiloride-sensitive $Na^+$ absorption by extracellular nucleotides in mouse trachea, Pflügers Arch. 444 (2001) 220–226.
[21] K. Kunzelmann, R. Schreiber, R. Nitschke, M. Mall, Control of epithelial $Na^+$ conductance by the cystic fibrosis transmembrane conductance regulator, Pflügers Arch. 440 (2000) 193–201.
[22] E.R. Lazarowski, L. Homolya, R.C. Boucher, T.K. Harden, Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation, J. Biol. Chem. 272 (1997) 24348–24354.
[23] C. Li, M. Ramjeesingh, C.E. Bear, Purified cystic fibrosis transmembrane conductance regulator (CFTR) does not function as an ATP-channel, J. Biol. Chem. 271 (1996) 11623–11626.
[24] M. Mall, M. Bleich, R. Greger, R. Schreiber, K. Kunzelmann, The amiloride inhibitable $Na^+$ conductance is reduced by CFTR in normal but not in CF airways, J. Clin. Invest. 102 (1998) 15–21.
[25] M. Mall, M. Bleich, R. Greger, M. Schurlein, J. Kühr, H.H. Seydewitz, M. Brandis, K. Kunzelmann, Cholinergic ion secretion in human colon requires co-activation by cAMP, Am. J. Physiol. 275 (1998) G1274–G1281.
[26] M. Mall, M. Bleich, J. Kühr, M. Brandis, R. Greger, K. Kunzelmann, CFTR mediated inhibition of amiloride sensitive sodium conductance by CFTR in human colon is defective in cystic fibrosis, Am. J. Physiol. 277 (1999) G709–G716.
[27] M. Mall, A. Wissner, J. Kühr, T. Gonska, M. Brandis, K. Kunzelmann, Inhibition of amiloride sensitive epithelial $Na^+$ absorption by extracellular nucleotides in human normal and CF airways, Am. J. Respir. Cell Mol. Biol. 23 (2000) 755–761.
[28] M. Mall, A. Wissner, H.H. Seydewitz, J. Kühr, M. Brandis, R. Greger, K. Kunzelmann, Defective cholinergic $Cl^-$ secretion and detection of $K^+$ secretion in rectal biopsies from cystic fibrosis patients, Am. J. Physiol. 278 (2000) G617–G624.
[29] L.J. Marshall, R. Muimo, C.E. Riemen, A. Mehta, $Na^+$ and $K^+$ regulate the phosphorylation state of nucleoside diphosphate kinase in human airway epithelium, Am. J. Physiol. 276 (1999) C109–C119.
[30] Y. Marunaka, N. Niisato, H. O'Brodovich, D.C. Eaton, Regulation of an amiloride-sensitive $Na^+$ permeable channel by a beta2-adrenergic agonist, cytosolic $Ca^{2+}$ and $Cl^-$ in fetal rat alveolar epithelium, J. Physiol. (Lond.) 515 (1999) 669–683.
[31] R. Muimo, S.J. Banner, L.J. Marshall, A. Mehta, Nucleoside diphosphate kinase and $Cl^-$ sensitive protein phosphorylation in apical membranes from ovine airway epithelium, Am. J. Respir. Cell Mol. Biol. 18 (1998) 270–278.
[32] S.J. Ramminger, A. Collett, D.L. Baines, H. Murphie, H.L. McAlroy, R.E. Olver, S.K. Inglis, S.M. Wilson, $P2Y_2$ receptor-mediated inhibition of ion transport in distal lung epithelial cells, Br. J. Pharmacol. 128 (1999) 293–300.
[33] M.M. Reddy, P.M. Quinton, C. Haws, J.J. Wine, R. Grygorczyk, J.A. Tabcharani, J.W. Hanrahan, K.L. Gunderson, R.R. Kopito, Failure of the cystic fibrosis transmembrane conductance regulator to conduct ATP, Science 271 (1996) 1876–1878.
[34] L.G. Rochelle, D.C. Li, H. Ye, E. Lee, C.R. Talbot, R.C. Boucher, Distribution of ion transport mRNAs throughout murine nose and lung, Am. J. Physiol. 279 (2000) L14–L24.
[35] E.M. Schwiebert, D.J. Benos, M.E. Egan, M.J. Stutts, W.B. Guggino, CFTR is a conductance regulator as well as a chloride channel, Physiol. Rev. 79 (1999) 8145–8166.
[36] E.M. Schwiebert, M.E. Egan, T.-H. Hwang, S.B. Fulmer, S.S. Allen, G.R. Cutting, W.B. Guggino, CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP, Cell 81 (1995) 1063–1073.
[37] E.M. Schwiebert, T. Flotte, G.R. Cutting, W.B. Guggino, Both CFTR and outwardly rectifying chloride channels contribute to cAMP-stimulated whole cell chloride currents, Am. J. Physiol. 266 (1994) C1464–C1477.
[38] M. Sugita, Y. Yue, J.K. Foskett, CFTR $Cl^-$ channel and CFTR-associated ATP channel: distinct pores regulated by common gates, EMBO J. 17 (1998) 898–908.
[39] K.J. Treharne, L.J. Marshall, A. Mehta, A novel chloride-dependent GTP-utilizing protein kinase in plasma membranes from human respiratory epithelium, Am. J. Physiol. 267 (1994) L592–L601.
[40] K.J. Treharne, A. Mehta, R. Muimo, NDPK, a protein kinase defective in CF binds to and is regulated by AMPK, a CFTR-associated protein, Pediatr. Pulmonol., Suppl. 22 (2001) 221 (Abstract).
[41] H.J. Veeze, M. Sinaasappel, J. Bijman, J. Bouquet, H.R. De Jonge, Ion transport abnormalities in rectal suction biopsies from children with cystic fibrosis, Gastroenterology 101 (1991) 398–403.
[42] T. Voelcker, B. Mürle, R. Schreiber, K. Kunzelmann, ENaC is inhibited by an increase in the intracellular $Cl^-$ concentration mediate rough activation of $Cl^-$ channels, Forefront in Nephrology Symposium, Monte Verita, Switzerland, 2002 (Abstract).
[43] W.C. Watt, E.R. Lazarowski, R.C. Boucher, Cystic fibrosis transmembrane regulator-independent release of ATP. Its implications for the regulation of $P2Y_2$ receptors in airway epithelia, J. Biol. Chem. 273 (1998) 14053–14058.