In vivo activation of CFTR-dependent chloride transport in murine airway epithelium by CNP

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Kelley, Thomas J., Calvin U. Cotton, and Mitchell L. Drumm. In vivo activation of CFTR-dependent chloride transport in murine airway epithelium by CNP. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1065–L1072, 1997.—Inhibitors of guanosine 3',5'-cyclic monophosphate (cGMP)-inhibited phosphodiesterases stimulate Cl− transport across the nasal epithelia of cystic fibrosis mice carrying the ΔF508 mutation [cystic fibrosis transmembrane conductance regulator (CFTR) (ΔF/ΔF)], suggesting a role for cGMP in regulation of epithelial ion transport. Here we show that activation of membrane-bound guanylate cyclases by C-type natriuretic peptide (CNP) stimulates hyperpolarization of nasal epithelium in both wild-type and ΔF508 CFTR mice in vivo but not in nasal epithelium of mice lacking CFTR [CFTR(−/−)]. With the use of nasal transepithelial potential difference (TEPD) assay, CNP was found to hyperpolarize lumen negative TEPD by 6.1 ± 0.6 mV in mice carrying wild-type CFTR. This value is consistent with that obtained with 8-bromoguanosine 3',5'-cyclic monophosphate (6.2 ± 0.9 mV). A combination of the adenylate cyclase agonist forskolin and CNP demonstrated a synergistic ability to induce Cl− secretion across the nasal epithelium of CFTR(ΔF/ΔF) mice. No effect on TEPD was seen with this combination when used on CFTR(−/−) mice, implying that the CNP-induced change in TEPD in CFTR(ΔF/ΔF) mice is CFTR dependent.

NATRIURETIC PEPTIDES are known regulators of fluid and ion transport in several systems (reviewed in Ref. 25), and have been shown to regulate Cl− transport in shark rectal gland cells (28) and in porcine colon via a guanosine 3',5'-cyclic monophosphate (cGMP)-dependent mechanism (1). Similarly, both heat-stable enterotoxin (Sta) and guanylin, peptides structurally and functionally related to the natriuretic peptides, have been shown to stimulate Cl− transport in the human colonic cell lines T84 and Caco-2 (3, 5) through a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent pathway.

The regulatory actions of the natriuretic peptides are thought to be mediated predominantly through cGMP. The natriuretic peptides bind to and activate particulate, transmembrane receptors that contain intracellular guanylate cyclase (GC) domains. Each of the natriuretic peptides, atrial (ANP), brain (BNP), and C-type (CNP), have varied affinities for specific receptors. ANP and BNP both bind to GC-A, with ANP having the greater affinity, and CNP binds to the GC-B receptor (reviewed in Ref. 32). Although there is an apparent specificity of function for ANP and BNP in cardiac tissue and in the pulmonary vascular system compared with CNP (9, 20, 21, 29), CNP binding and function predominates in airway epithelial cells (6, 7, 30). Specific binding of CNP to human airway epithelial cells has been demonstrated by the functional ability of CNP to elevate cGMP levels in these cells. ANP had no significant effect on cGMP levels, although sodium nitroprusside (SNP), an activator of soluble GCs, did elevate cGMP concentrations in this system. Additionally, CNP was shown to increase ciliary beat frequency in primary airway epithelial cells, implying a role for CNP in the regulation of mucociliary clearance (6) and raising the possibility that CNP may play a role in the regulation of other nonrespiratory functions in the airway.

We have previously shown that CNP may play a role in regulating Cl− secretion in cultured airway epithelial cells, the possible paracrine or autocrine regulation of CFTR activity by natriuretic peptides has not been established. The first step in this process is to demonstrate stimulation of CFTR in intact tissue by CNP. We have chosen the mouse as a model in which to study this system because the CNP gene is conserved between mice and humans (22, 23) and because CFTR null [CFTR(−/−)] mice are available to serve as controls for CNP-mediated CFTR activation (2, 8, 17, 26). In this paper, in vivo mouse nasal transepithelial potential difference (TEPD) measurements as well as in vitro mouse tracheal TEPD measurements were made in response to apically applied CNP and other GC agonists to demonstrate their ability to regulate Cl− transport across murine respiratory epithelium.

MATERIALS AND METHODS

Peptides. Both CNP and vasonatrin were purchased from Bachem Bioscience. Measurement of mouse nasal TEPD values. Mouse nasal TEPD was measured by the protocol of Grubb et al. (8).
Briefly, mice were anesthetized with 200 µl/20 g body wt of 0.4 mg acepromazine, 11 mg ketamine, and 2 mg xylazine/ml phosphate-buffered saline. PE-10 tubing drawn out to approximately one-half of its original diameter was inserted 2–3 mm into the nostril of the mouse. Solutions were perfused at room temperature with the use of a Razel A-99 (Razel Scientific Instruments, Stamford, CT) syringe pump at a rate of 7 µl/min. Each syringe was bridged to a Calomel electrode through a 4% agar bridge made up in either Cl\(^{2-}\)-replete or Cl\(^{2-}\)-free Ringer solution. An 10-mV junction potential in the negative direction was instantly seen upon switching to Cl\(^{2-}\)-free solutions. This junctional potential is not shown in Figs. 1–9 and is not corrected for in the values given. A series of valves was used to change solutions, with a delay time of 45 s between solution change and solution contact with the nasal epithelia. Ringer solutions consisted of Cl\(^{2-}\)-replete N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered Ringer solution [HBR; containing (in mM) 10 HEPES (pH 7.4), 138 NaCl, 5 KCl, 2.5 Na\(_2\)HPO\(_4\), 1.8 CaCl\(_2\), and 1.0 MgSO\(_4\)] and Cl\(^{2-}\)-free HBR [containing (in mM) 10 HEPES (pH 7.4), 138 sodium gluconate, 5 potassium gluconate, 2.5 Na\(_2\)HPO\(_4\), 3.6 hemicalcium gluconate, and 1.0 MgSO\(_4\); all chemicals were from Sigma Chemical, St. Louis, MO]. Some mice were studied on multiple occasions. Mice that were retested were given at least 48 h between assays.

**Measurement of mouse tracheal potential difference values.**

Excised trachea were mounted on hold pipettes (30-ml micropipettes; Drummond Scientific) inserted into each end of the trachea, and the luminal and bath compartments were perfused independently. All experiments were performed at 37°C by placing all solutions and the trachea-mounting chamber in an Isolette infant incubator (Narco, Hatboro, PA). HBR was perfused to both luminal and basolateral sides by gravity. Luminal perfusion rates were between 3 and 5 ml/min. TEPD was measured via 4% agar bridges in HBR placed on both luminal and basal sides and was connected through calomel
electrodes to a DVC 1000 voltage-current clamp (WPI). Data were collected on a MacLab/4e from Advanced Instruments.

cGMP measurements. Mouse tracheal tissue was excised and cut into three equal sections for each experiment. The epithelial layer was not isolated for these experiments, so various cell types were present, including neuronal cells. The tissue was incubated for 5 min at 37°C in physiological salt solution buffered by 10 mM tris(hydroxymethyl)aminomethane (Tris; pH 7.5) containing either 1 µM CNP or 300 µM SNP or in the absence of any GC agonist as a control. Samples were then placed in 0.5 ml of 10 mM Tris (pH 7.5) and 100 µM EDTA at 90°C for 10 min. Samples were then placed on ice, homogenized, and spun down in a microfuge at 14,000 revolutions/min for 5 min, and the supernatant was collected for analysis. Measurements of cGMP levels were performed using an enzyme-linked immunoassay kit, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Mice. Mice were genotyped from tail clip DNA. DF508 mice were a generous gift from Kirk Thomas of the University of Utah School of Medicine and were genotyped by the procedures described previously (33). CFTR(+/−) mice (27) were obtained from Jackson Laboratories and were genotyped as described by Koller et al. (16). To increase survival of cystic fibrosis animals, mice were fed a liquid diet as described by Eckman et al. (4). Mice were cared for in accordance with Case Western Reserve University guidelines.

RESULTS

Effects of cGMP agonists on mouse nasal TEPD. Three GC agonists and a cGMP analog, 8-bromoguanosine 3’5’-cyclic monophosphate (8-BrcGMP), were tested by nasal TEPD assay for their ability to stimulate Cl− secretion in the nasal epithelia of mice when perfused onto murine nasal epithelia in Cl−-free HBR (Fig. 1). An agonist of cytosolic GCs, SNP (300 µM), had very little effect on TEPD values, hyperpolarizing lumen negative TEPD only 2.4 ± 0.9 mV (n = 7). In contrast, 1 µM CNP, which activates GC-B, a membrane-bound GC, stimulates a change in TEPD (ΔTEPD) of 6.1 ± 0.6 mV (n = 8), representing lumen negative hyperpolarization. Similarly, the GC-B-specific peptide...
vasonatrin (1 µM), a chimeric peptide that contains the bioactive loop region of CNP, and the carboxy-terminus of ANP hyperpolarized lumen negative TEPD 6.5 ± 1.1 mV (n = 4). Consistent with these effects being mediated by increased cGMP production, addition of the analog 8-BrcGMP (100 µM) resulted in a ΔTEPD of 6.2 ± 0.9 mV (n = 3).

Pharmacological characterization of CNP-mediated Cl− secretion in mouse nasal epithelia. Previously, we reported that CNP stimulated CFTR-mediated Cl− current in Calu-3 cells as determined by whole cell patch-clamp recordings (13). To determine if CFTR has a role in CNP-stimulated Cl− secretion in vivo, the Cl− channel blockers 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and diphenylamine-2-carboxylate (DPC) were used to pharmacologically characterize responses to CNP (18, 31). DPC (1 mM) reduced CNP-stimulated changes in mouse nasal TEPD, 70%, whereas 500 µM DIDS did not have a significant effect (Fig. 2). This inhibitor sensitivity profile is consistent with CFTR activation. For comparison, forskolin-stimulated nasal TEPD changes were also tested in the presence of DPC and DIDS. Forskolin-stimulated ΔTEPDs were reduced ~55% by DPC and were unaffected by the presence of DIDS (Fig. 2B).

To more directly test for CFTR activity in response to CNP, CFTR(−/−) mice were compared with wild-type mice in their ability to respond to CNP. CNP (1 µM), in Cl−-free HBR and in the presence of amiloride, was perfused into the nasal cavity of CFTR(−/−) mice as described for the wild-type mice. The CFTR(−/−) mice failed to show any hyperpolarization of lumen negative potential in response to either Cl−-free HBR alone or with the agonist CNP (Fig. 3). These data, coupled with the inhibitor profile of CNP-induced changes in TEPDs, are strong evidence that CNP stimulates epithelial Cl− transport by regulating CFTR activity.

Fig. 4. Elevation of cGMP values in response to either 300 µM SNP or 1 µM CNP in trachea of CFTR(+/−) or CFTR(+/+) mice. Determinations of cGMP content were performed as described in MATERIALS AND METHODS. Error bars represent SE, and n is shown in parentheses.

To show that CNP was acting through its receptor GC-B, two inhibitors of GC activity were used (Fig. 4). First, perfusion with the compound LY-83583 (50 µM), which directly inhibits GC activity (15, 19), completely inhibited any response to CNP. Second, perfusion with 250 nM phorbol 12,13-dibutyrate (PDBu) inhibited CNP-mediated changes in TEPD by ~90%, consistent with reports that phorbol esters cause protein kinase (PK) C-dependent desensitization of natriuretic peptide receptors (24). For comparison, PDBu had no effect on forskolin-stimulated changes in TEPD, indicating that inhibition of the CNP effect was not due to PDBu interfering the ability of CFTR to activate.

Fig. 5. Elevation of cGMP values in response to either 300 µM SNP or 1 µM CNP in trachea of CFTR(+/+) or CFTR(+/−) mice. Determinations of cGMP content were performed as described in MATERIALS AND METHODS. Error bars represent SE, and n is shown in parentheses.

Fig. 6. In vitro TEPD changes of a wild-type mouse trachea in response to Forsk (Fsk) and CNP. A: raw trace of tracheal TEPD assay showing responses to luminal applications of 100 µM amiloride (A), 10 µM Fsk, and 1 µM CNP. HBR represents perfusion with Cl−-replete HEPES-buffered Ringer solution. Reduction (depolarization) in TEPD caused by amiloride is partially restored by the addition of Fsk. After washout with HBR, the TEPD returns (hyperpolarizes) to baseline. Treatment with amiloride again depolarizes the TEPD, and CNP has no appreciable effect. After a second washout and depolarization with amiloride, Fsk is added a second time. Basal TEPD values for these experiments were 2.8 ± 0.3 mV (n = 4). B: bar graph showing ΔTEPD in response to the first addition of Fsk alone (Fsk1; n = 3), CNP alone (CNP; n = 4), Fsk plus CNP (Fsk+CNP; n = 3), and a second addition of Fsk (Fsk2; n = 3). Significance is measured against responses to Fsk. Positive ΔTEPD values indicate a hyperpolarization of lumen negative potential difference. Error bars represent SE. Statistical analysis is a comparison of the responses to the indicated agents with the responses to Fsk. Duncan’s multiple range test with a Bonferroni correction for multiple comparisons was used. Significance at α = 0.05 corresponds to P < 0.017. *P < 0.0005.
Stimulation of Cl\(^{-}\) secretion in mouse trachea by CNP. To determine if CNP acts on epithelia in other portions of the airway, the effects of CNP on Cl\(^{-}\)-transport were tested by measuring TEPD values in excised mouse trachea. We first established that the CNP receptor was present and functional in tracheal tissue by measuring cGMP production in response to 1 µM CNP and 300 µM SNP (Fig. 5). Compared with tissues not incubated with GC agonists, SNP increased cGMP levels ~2-fold, whereas CNP increased cGMP levels ~50-fold.

Whereas the nasal TEPD assays were performed by perfusing Cl\(^{-}\)-free Ringer solution onto the surface of murine nasal epithelium to increase the electrochemical driving force for Cl\(^{-}\) secretion, the tracheal TEPD assays were performed with Cl\(^{-}\)-replete Ringer solution on both the basolateral and luminal sides. Under these conditions, CNP alone was not sufficient as a

Fig. 7. Stimulation of CFTR-dependent ΔTEPDs in mouse nasal epithelia. A: averaged traces of nasal TEPD assays performed on CFTR(ΔF/ΔF) mice treated with either 10 µM Forsk (n = 3), 1 µM CNP (n = 3), or 10 µM Forsk plus 1 µM CNP (n = 8). Zero ΔTEPD and time 0 correspond to the point at which agonists were added. All traces were performed in the presence of 100 µM amiloride in Cl\(^{-}\)-free HBR. Positive ΔTEPD values indicate a hyperpolarization of lumen negative potential difference. Error bars represent SE. B-D: raw traces of Forsk alone (B), CNP alone (C), and Forsk plus CNP (D). Agonists were added at the 2-min time point (arrows).

Fig. 8. Averaged traces of nasal TEPD assays performed on CFTR(ΔF/ΔF) mice (n = 8) or CFTR(−/−) mice (n = 4) treated with 10 µM Forsk plus 1 µM CNP. Values were taken at 15-s intervals and were plotted as a ΔTEPD. Zero ΔTEPD and time 0 correspond to the point at which agonists were added. All traces were performed in the presence of 100 µM amiloride in Cl\(^{-}\)-free HBR. Positive ΔTEPD values indicate a hyperpolarization of lumen negative potential difference. Error bars represent SE. B and C: raw traces of CFTR(ΔF/ΔF) mice and CFTR(−/−) mice treated with Forsk plus CNP, respectively. Agonists were added at the 2-min time point.
stimulus to generate hyperpolarization in tracheal epithelium and resulted in further depolarization of TEPD. Forskolin alone did generate a detectable hyperpolarization of TEPD in the trachea. However, a combination of forskolin plus CNP stimulated a twofold greater ΔTEPD than forskolin alone (Fig. 6). This synergistic relationship between forskolin and CNP suggests an enhancement of the adenosine 3′,5′-cyclic monophosphate-PKA pathway rather than additive effects of two separate pathways.

In vivo activation of ΔF508 CFTR by forskolin and CNP. We have previously shown that Cl− transport can be stimulated in the nasal epithelium of ΔF508/ΔF508 mice with the combination of forskolin and the cGI-PDE inhibitor milrinone but not with either compound alone (14). Activation of Cl− secretion was not observed when nasal epithelium of CFTR(−/−) mice was exposed to these compounds. Our hypothesis from these data is that both CNP and milrinone are acting to stimulate CFTR activity by inhibition of cGI-PDEs. If true, the combination of forskolin and CNP, like forskolin and milrinone, should stimulate Cl− secretion in ΔF508/ΔF508 mice.

Mouse nasal TEPD assays with ΔF508/ΔF508 mice showed that neither forskolin nor CNP alone stimulates a hyperpolarization of TEPD, as we had found with milrinone alone. Consistent with our hypothesis, after a 2-min exposure to forskolin plus CNP, TEPD was hyperpolarized 3.4 ± 1.0 mV (n = 8) compared with a depolarization of 2.1 ± 0.6 mV (n = 3) induced by forskolin or a 2.1 ± 0.9 mV (n = 3) depolarization induced by CNP alone (Fig. 7). This 3.4 ± 1.0-mV hyperpolarization of TEPD in CFTR(ΔF/ΔF) mice is ~35% of the amount of activation achieved with the combination of forskolin and CNP in CFTR(+/ΔF) mice (9.7 ± 1.3 mV; n = 3), whereas forskolin plus CNP induced a 2.3 ± 0.8-mV (n = 4) depolarization of TEPD in CFTR(−/−), implicating a role for CFTR in the response to this combination of compounds. One differential response of CFTR(−/−) mice and CFTR(ΔF/ΔF) mice is illustrated in Fig. 8. These data show that CFTR is involved in the mechanism of CNP-induced Cl− secretion in airway epithelia of mice.

DISCUSSION

We have previously shown that cGI-PDEs are an important part of the CFTR regulatory pathway (11, 12, 14). The involvement of this PDE class suggests an important role for cGMP in the normal regulation of Cl− transport across CFTR-expressing epithelium. This notion is supported by other reports that show CFTR activation in human colonic cells in response to both Sta and guanylin, both of which are peptides that activate membrane-bound GCs (3, 5). A similar peptide, CNP, has been shown to stimulate cGMP production in primary cultures of human airway epithelial cells (7).

We have demonstrated in cultured lung epithelial cells (Calu-3) that CNP is capable of stimulating CFTR activity as shown by whole cell patch-clamping experiments (13). This stimulation of activity was mediated by cGMP but via a PKA-dependent pathway, consistent with our previous results from the cGI-PDE inhibitor milrinone.

Natriuretic peptides have been shown to regulate fluid and electrolyte transport in several systems (1, 19, 25, 28). The ability of CNP to stimulate cGMP production in human airway epithelial cells suggests a role for CNP in the regulation of airway electrolyte transport. Our data show that CNP can stimulate Cl− secretion when applied to the apical membrane of mouse nasal epithelia in vivo. This stimulated Cl− transport has the pharmacological characteristics of CFTR and is not present when CNP is applied to the nasal epithelium of CFTR(−/−) mice.

It has also been reported, however, that CNP has no effect on Cl− transport when applied to cultured monolayers of ciliated human nasal epithelial cells obtained from nasal scrapings (6). A possible reason for this discrepancy is that culture conditions for primary cells are not optimal for facilitating responses to CNP, thus reducing reproducibility and magnitude of CNP-mediated stimulation. Thus our in vivo measurements of nasal TEPD and our in vitro studies with freshly excised trachea may provide a more appropriate model than studies with cultured cells. A methodological difference between the studies is that our nasal TEPD measurements were made with the luminal side of the epithelium perfused with Cl−-free HBR, whereas the short-circuit current measurements made in the previous report were in symmetrical Cl−-replete Krebs-buffered Ringer (KBR). The short-circuit data are consistent with our findings in freshly excised trachea in KBR (Fig. 6) in which we were unable to see a response with CNP in the presence of amiloride. We could demonstrate, however, a synergistic increase in forskolin-mediated hyperpolarization of TEPD by CNP. Another possible difference between systems may lie in the fact that CNP and its receptor GC-B have been
identified in brain tissue and nerve fibers (reviewed in Ref. 10). It is possible that in our in vivo system CNP is interacting with nerve fibers within the nasal passage and stimulating the release of other factors that may be initiating CFTR activation in the epithelium. This pathway would not be available in cultured monolayers of harvested primary epithelial cells. We think this is unlikely to explain the difference seen here, as our previous study in Calu-3 and CF-T43 cells showed that cultured epithelial cells exposed to CNP are sufficient to stimulate CFTR-dependent Cl\(^{-}\) transport (13).

The demonstrated ability of natriuretic peptides to generically regulate salt and fluid transport in several systems opens the possibility that an in vivo role of CNP may be to regulate Cl\(^{-}\) transport in the airways through CFTR. The similarity of effects that both cGIPDE inhibitors and the GC agonist CNP have on CFTR activity implies a regulatory role for cGMP in ion transport regulation across airway epithelial cells. The specificity of CNP-mediated regulation of CFTR activity extends to its ability to stimulate Cl\(^{-}\) transport across nasal epithelia of CFTR\(\Delta F/\Delta F\) mice when used in combination with the adenylyl cyclase agonist forskolin but has no effect on Cl\(^{-}\) secretion in CFTR\((-/-)\) mice. These results are consistent with our previous data demonstrating that the combination of forskolin and the cGIPDE inhibitor milrinone stimulates CFTR activity in the nasal epithelia of \(\Delta F/\Delta F\) mice and support the hypothesis that milrinone is acting via a cGMP-dependent pathway (Fig. 9). The ability to stimulate Cl\(^{-}\) secretion in the nasal epithelium of \(\Delta F/\Delta F\) mice in an in vivo system demonstrates the therapeutic potential of CNP as a pharmacological agent and the possible importance of CNP as an in vivo regulator of CFTR function.

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REFERENCES


