Mechanisms of Cystic Fibrosis Transmembrane Conductance Regulator Activation by S-Nitrosothiogluthione*

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a 1480-amino acid protein and a member of the traffic ATPase family (1). It functions as a cAMP-regulated Cl− channel (2) and controls other ion conductive pathways, including epithelial Cl− and Na+ channels (3, 4). In most epithelial cells, phosphorylation of the CFTR regulatory domain by cAMP-dependent protein kinase A increases its activity as a Cl− channel (5). Although earlier studies localized CFTR mainly in airway cells, more recent studies provide convincing evidence for the presence of both immunoreactive and functional CFTR at the apical membranes of both fetal and adult ATII cells (6).

There is considerable evidence that reactive oxygen nitrogen species, generated by environmental pollutants and activated inflammatory or airway cells, may alter the properties of a number of ion channels either by direct modification (such as nitrosation, nitration, and oxidation) or via signal transduction pathways (7). Our previous studies indicate that prolonged exposure of confluent monolayers of Calu-3, 16 human bronchial epithelial, or mouse tracheal epithelial cells to physiological levels of NO, generated by the chemical donor DETA NONOate, decrease levels of CFTR in their apical membranes and impaired chloride (Cl−) secretion in response to cAMP, a well established regulator of CFTR (8, 9). These changes were found to be due to post-translational modification of CFTR by reactive oxygen nitrogen intermediates. Decreased levels and function of normal CFTR may account for some of the cystic fibrosis-like symptoms that occur in chronic inflammatory lung diseases associated with increased NO production.

In several systems, the biological effects of NO on transport proteins have been associated with the formation of nitrosothiols (7). Micromolar concentrations of S-nitrosothiogluthione (GSNO) have been detected in the airway fluid of normal subjects, and substantially higher levels were observed in the lungs of patients with pneumonia or during inhalation of 80 ppm of NO (10). Conversely, lower concentrations of GSNO were found in the lower airways of patients with cystic fibrosis (11) and asthma (12). GSNO may directly trans-nitrosate proteins, or release NO via reduction or homolysis. Oxidized glutathione may oxidize or glutathionylate a number of proteins, events known to alter protein function. NO, generated from GSNO, can stimulate cGMP or react with superoxide to form peroxynitrite (ONOO−), providing an alternate pathway for directly modulating ion channel function (13). More recently, amino acid transporters have been identified as potential mediators of S-nitrosothiol transport without the intermediate formation of NO, revealing a novel mechanism through which S-nitrosothiol metabolism is controlled (14).

Currently, the effects of GSNO on CFTR function are in dispute: Zaman et al. (15) reported that GSNO increases maturation and function of both wild-type and AF508 CFTR via post-transcriptional regulation, most likely via S-nitrosation. However, Wang et al. (16) reported that oxidized forms of glutathione (including GSNO) decreased CFTR function by oxidative modifications (gluthionylation). However, none of these studies utilized confluent monolayers of airway cells and thus were unable to ascertain the effects of GSNO on vectorial Cl− transport.

Herein we exposed Calu-3 cells, an extensively used model of human airway gland serous cells, grown under an air liquid interface, to physiological concentrations of GSNO for up to 6 h and measured Cl− short circuit currents (Isc) and trans-epithelial resistance (Rt). We have chosen to use Calu-3 cells, because they do not contain epithelial sodium channels, which are also affected by NO adducts (17–19), complicating the interpretation of the results. Our results indicate that addition of GSNO into the apical compartment of Ussing chambers, containing either intact or basolaterally permeabilized monolayers of Calu-3, resulted in sustained increases of Isc without significant changes of Rt. These...
increases were partly due to (i) stimulation of soluble guanylyl cyclase and possible phosphorylation of CFTR via cGMP-dependent mechanisms and (ii) nitrosation of CFTR or other chaperon proteins by reactive intermediates of GSNO and cysteine (S-nitroscysteine), entering the cytoplasm via amino acid transport system L, where they are capable of nitrosating proteins without the intermediate formation of NO (20).

MATERIALS AND METHODS

Cell Culture—Human airway mucosal gland cells Calu-3 cells (HTB-55, ATCC, Manassas, VA) were grown using minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and maintained in T-75 tissue culture flasks (Costar, Cambridge, MA) at 37 °C in a humidified atmosphere of 5% CO2 in air. Confluent cell layers were washed with phosphate-buffered saline two times and then incubated with 0.05% trypsin and 0.53 mM EDTA in saline for 30 min to lift them from the flasks. Cells were pelleted by centrifugation (125 × g for 10 min), resuspended in the culture medium, and seeded at a density of 106 cells/cm2 onto Type 3470 Costar Transwell inserts (Corning Inc., Corning, NY, 0.45-µm pore size, 0.33-cm2 surface area, 0.4 µm), for mounting in Ussing chambers. The culture media on the basolateral side of the filters was replaced every 48 h. After 3 days, the apical fluid was removed, and when monolayers were able to exclude fluid for the apical surface they were cultured for an additional 3–4 days using an air (apical) and fluid (basolateral) interface (21). They were then used for bioelectric studies as described below.

NO Donors and Inhibitors—GSNO (Alexis, San Diego, CA) was dissolved in phosphate-buffered saline containing 10 µM diethylenetriaminepentaacetic acid (Acros, Fairlawn, NJ). GSNO concentrations were determined by measuring the absorbance of the stock solution at 333 nm (ε336 nm = 900 mM⁻¹ × cm⁻¹) with a Beckman DU 7400 spectrophotometer (Beckman Coulter, Fullerton, CA). Oxy-myoglobin (oxy-Mb) was prepared as previously described (22). Briefly, myoglobin from horse heart (Sigma) was dissolved in phosphate-buffer saline containing 10 µM diethylenetriaminepentaacetic acid. Met-myoglobin was reduced to oxy-Mb by addition of sodium hydroxysulfite and subsequently purified by gel-filtration chromatography using an NAPT 10 column (Amersham Biosciences). Oxy-Mb concentrations were determined by a Beckman DU 7400 spectrophotometer (Beckman Coulter) using the extinction coefficient per heme group ε582 nm = 14.4 mM⁻¹ × cm⁻¹. Diethylyamine NONOate (DEA/NO) (t½ = 2 min at 37 °C) was purchased from Cayman Chemical (Ann Arbor, MI) and prepared fresh each day according to manufacturer’s instructions.

Measurements of Nitrosothiols and NO Release—GSNO (10 µM) was added into the apical compartment of an Ussing chamber housing with either confluent monolayer of Calu-3 cells or empty filters. The chambers were filled with a solution containing (in mM) 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.83 KHPO4, 1.2 CaCl2, 1.2 MgCl2, 10 HEPES (Na+-free), 10 mannitol (apical compartment), and 10 glucose (basolateral compartment). Upper panel, NO concentrations measured using an ISO-NO electrode. Results of a typical experiment are shown, which was repeated three times. The thin arrows indicate the time of addition of GSNO (10 µM), whereas short thick arrows indicate addition of boluses of oxygen-Mb (0.1, 1, and 10 µM) into the apical compartments of the Ussing chambers. Lower panel, values of S-nitrosothiol concentrations in the apical compartment fluid measured as described in the text. Values are means ± S.E. (n = 3).

Measurements of Short-circuit Current (Isc) and Electrical Resistance (Rt)—Monolayers of Calu-3 cells were mounted in Ussing chambers (Jim’s Instruments, Iowa City, IA) and bathed on both sides with solutions containing: (mM) 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.83 KHPO4, 1.2 CaCl2, 1.2 MgCl2, 10 HEPES (Na+-free), 10 mannitol (apical compartment), and 10 glucose (basolateral compartment). Osmolarity of all solutions, as measured by a freezing depression osmometer (Wescor Inc., Logan, UT) was between 290 and 300 mosm. Bath solutions were stirred vigorously by bubbling continuously with 95% O2, 5% CO2 at 37 °C (pH 7.4). Monolayers were short-circuited to 0 mV and short-circuit currents (Isc) were measured with an epithelial voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA). A 10-mV pulse of 1-s duration was imposed every 10 s to monitor Rs, which was calculated using Ohm’s law. Data were collected using the Acquire and Analyze program, version 1.45 (Physiologic Instruments).

To ascertain the contribution of Cl⁻ and HCO3⁻ ions to the baseline and GSNO, induced Isc, NaCl, or NaHCO3 were replaced with equimolar amounts of Na+-gluconate or Na+-HEPES, respectively. In a third set of ion substitution experiments, both Cl⁻ and HCO3⁻ were replaced with Na+-gluconate and Na+-HEPES. In experiments conducted in the absence of HCO3⁻ solutions were bubbled with 100% O2 instead of 95% O2 and 5% CO2.

Once Isc reached steady-state values (usually within 10–20 min from mounting), GSNO (0.1–200 µM) was added into the apical compartment and Isc and Rt were recorded till they reached new steady-state values. At that time, the CFTR blockers glibenclamide (0.1–0.4 mM, Sigma) or CFTR inh-172 (20 µM, Calbiochem), oxy-Mb (0.1–400 µM), or two protein phosphatases PP2A1 and PP2A2 (2 milliunits/ml, Calbiochem) were added into the apical compartments and changes in Isc.
and $R_t$ were recorded. In some experiments, Calu-3 cell monolayers were pre-treated with ODQ (5 mM, Tocris Cookson, St. Louis, MO), a specific inhibitor of soluble guanylate cyclase, every 12 h for 72 h prior to being mounted in the Ussing chambers.

To evaluate effects of GSNO on apical membrane Cl$^-$ conductance, Calu-3 monolayers were mounted in Ussing chambers under short-circuit conditions in the presence of a basolateral to apical (125:5 mM) Cl$^-$ gradient, and the pore-forming antibiotic amphotericin (10 mM) was added into the basolateral compartment. Under these conditions, the basolateral membrane is eliminated as a barrier to the flow of monovalent ions, and $I_{sc}$ provides a direct measure of the apical membrane Cl$^-$ conductance. GSNO (10 μM) was then added into the apical compartment followed by glibenclamide or CFTR inh-172.

**Measurements of cAMP and cGMP**—Filters containing Calu-3 cells were mounted in Ussing chambers; GSNO (10 μM), along with cysteine (50 μM) or an equivalent amount of vehicle, were added into the apical compartment for either 3 or 30 min. At the end of these times, filters were incubated with 0.1 M HCl plus 1% Triton X-100, and cells were pelleted by centrifugation at 600 $g$ and kept at $-80^\circ$C. cAMP or cGMP levels were measured using the Direct cAMP or cGMP enzyme-immunoassay kits (Assay Designs Inc., Ann Arbor, MI), according to the manufacturer’s specifications.

**Statistical Analysis**—All data were expressed as means ± 1 S.E.; statistical significance among multiple means was assessed by analysis of variance, followed by the Bonferroni modification of the $t$ test, adjusted for multiple comparisons; $p$ values of < 0.05 were considered significant.

**RESULTS**

**Generation of NO**—Addition of 10 μM GSNO into the apical compartment of an Ussing chamber containing empty filters resulted in the immediate formation of NO up to ~1 μM, which then declined to a steady-state value of ~0.3 μM, lasting over 45 min. Addition of 10 μM oxy-Mb into the apical compartment decreased the concentration of NO to non-detectable levels. Smaller concentrations oxy-Mb (0.1–1 μM) also decreased NO levels but in a transient fashion (Fig. 1). In contrast, when 10 μM GSNO was added into Ussing chambers contain-
**TABLE 1**

GSNO (10 μM) increases I\textsubscript{sc} across confluent monolayers of Calu-3 cells mounted in Ussing chambers

|                               | Baseline (M GSNO) | Peak (M GSNO) | Plateau (M GSNO) | ΔPeak | ΔPlateau | n  |
|-------------------------------|-------------------|---------------|------------------|-------|----------|----|
| Vehicle                       | 8.5 ± 0.7         | 8.4 ± 0.8     | 10.0 ± 1.0       | −0.1  | 1.5 ± 0.4 | 34 |
| GSNO (10 μM)                  | 8.7 ± 0.4         | 20 ± 0.7\a    | 14.4 ± 0.6\a     | 11.0  | 5.7 ± 0.3\a | 198 |

1) Effects of CFTR inhibitors added apically post GSNO

|                               | Baseline (M GSNO) | Peak (M GSNO) | Plateau (M GSNO) | Glib. admin. | Glib. admin. | CFTRinh.–172 admin. | ΔPeak | ΔPlateau | ΔPlateau | n  |
|-------------------------------|-------------------|---------------|------------------|--------------|--------------|---------------------|-------|----------|----------|----|
| GSNO (10 μM)                  | 7.0 ± 0.9         | 17 ± 1.8\a    | 11 ± 1.6\a       | 5.6 ± 0.9    | 4.0 ± 1.0    | 10                  | 5.4 ± 1.7 | 10 |
| GSNO (10 μM)                  | 7.8 ± 0.7         | 17.1 ± 3\b    | 10.5 ± 0.5\b     | 8.6 ± 0.3    | 2.7 ± 0.8\b  | 3                   | 1.9 ± 0.2 | 3  |

2) Effects of ouabain added basolaterally post GSNO

|                               | Baseline (M GSNO) | Peak (M GSNO) | Plateau (M GSNO) | Ouab. admin. | ΔPeak | ΔPlateau | n  |
|-------------------------------|-------------------|---------------|------------------|--------------|-------|----------|----|
| GSNO (10 μM)                  | 12.4 ± 1.4        | 21 ± 3\a      | 15.5 ± 2.5\a     | 0.76 ± 2.4   | 3.1 ± 1.3\b | 5                   | 14.7 ± 3   | 5   |

3) Effects of pre-treatment with glibenclamide on the GSNO response

|                               | Baseline (M GSNO) | Glib. admin. | Peak (M GSNO) | Plateau (M GSNO) | ΔPeak | ΔPlateau | n  |
|-------------------------------|-------------------|--------------|---------------|------------------|-------|----------|----|
| GSNO (10 μM)                  | 9.4 ± 1.2         | 7.2 ± 1.5    | 11.5 ± 2.1    | 7.2 ± 1.4       | 0.03 ± 0.5 | 6   |

\a p < 0.05 compared to each corresponding baseline value (analysis of variance followed by modified t-test).

\b p < 0.05 compared to the corresponding vehicle value (t-test).

**FIGURE 3. Effects of GSNO on I\textsubscript{sc} of Calu-3 cells bathed with Cl\textsuperscript{−} and HCO\textsubscript{3}\textsuperscript{−} free solutions.** Confluent monolayers of Calu-3 cells were mounted in Ussing chambers containing normal Ringer’s solution (NR), Cl\textsuperscript{−} free (−/− Cl\textsuperscript{−}), HCO\textsubscript{3}\textsuperscript{−} free (−/− HCO\textsubscript{3}\textsuperscript{−}), and both Cl\textsuperscript{−} and HCO\textsubscript{3}\textsuperscript{−} free (−/− Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−}) free solutions (see ‘Materials and Methods’). GSNO (10 μM) was added into the apical compartments of the Ussing chambers, and peak and steady-state responses were recorded. Glibenclamide (Glib., 0.3 mM) was added into the apical compartments once steady-state values of I\textsubscript{sc} were achieved. Val- ues are means ± S.E. The number of monolayers for each group was as follows: NR = 35; (+) Cl\textsuperscript{−} = 25; (+) HCO\textsubscript{3}\textsuperscript{−} = 14; and (+) Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} = 4. * p < 0.05 compared with their corresponding baseline values (by pared t-tests).

GSNO Increases Cl\textsuperscript{−} Secretion across Calu-3 Cells—Addition of 10 μM GSNO into the apical compartment containing Calu-3 cells resulted in an immediate transient increase of I\textsubscript{sc} followed by a sustained plateau (Fig. 2). Mean values (± S.E.) are shown in Table 1. The sustained increase of Cl\textsuperscript{−} secretion was reversed by the apical addition of the CFTR inhibitors glibenclamide (0.3 mM, Fig. 2) or CFTRinh.–172 (20 μM, Fig. 2) or the basolateral addition of ouabain (1 mM, Table 1). Transepithelial resistance decreased somewhat, but stabilized to >600 Ω · cm\textsuperscript{2} (Fig. 2). Addition of glibenclamide into the apical compartment prior to GSNO decreased I\textsubscript{sc} and prevented the sustained increase of I\textsubscript{sc} in response to GSNO (see Table 1). The IC\textsubscript{50} for the GSNO-induced increase of I\textsubscript{sc} was 3.6 ± 1.3 μM (n = 6) (Fig. 2). Corresponding amounts of vehicle, nitrite, GSSG, or GSH had no effect on I\textsubscript{sc} or R\textsubscript{t} (data not shown).

Because Calu-3 cells transport both Cl\textsuperscript{−} and HCO\textsubscript{3}\textsuperscript{−}, we investigated the contribution of both ions to the transient and steady response of the I\textsubscript{sc} to GSNO. As seen in Fig. 3, the majority of the peak increase was due to movement of HCO\textsubscript{3}\textsuperscript{−}, whereas both Cl\textsuperscript{−} and HCO\textsubscript{3}\textsuperscript{−} contributed equally to the steady-state increases.

GSNO Increases I\textsubscript{sc} by Stimulating CFTR—Because Cl\textsuperscript{−} secretion across Calu-3 cells involves the coordinated entry of Cl\textsuperscript{−} into the cytoplasm via basolateral transporters and their subsequent secretion across the apical membrane through CFTR pathways, we investigated the effects of GSNO across basolaterally permeabilized monolayers in the presence of secretory Cl\textsuperscript{−} gradients. As shown in Fig. 4 and Table 2, GSNO increased I\textsubscript{sc} in a manner similar to what was observed in intact monolayers. Addition of glibenclamide (Fig. 4), oxy-Mb (Fig. 4) or CFTRinh.–172 (Table 2) into the apical compartments returned I\textsubscript{sc} to its pre-GSNO levels.

Role of NO in GSNO-dependent Stimulation of CFTR—As shown in Fig. 5, addition of oxy-Mb (1 μM) into an apical chamber decreased the GSNO-induced I\textsubscript{sc} by <20%. At higher concentrations (300 μM), oxy-Mb either completely prevented or immediately decreased the GSNO increase of I\textsubscript{sc} (Fig. 5 and Table 3). This was not due to nonspecific inhibition of CFTR, because the subsequent addition of 10 μM forskolin readily increased I\textsubscript{sc} to the same extent as in control cells (Fig. 6). These data indicate that NO must be present to stimulate Cl\textsuperscript{−} secretion. Indeed, as shown in Fig. 6 addition of diethylenemine NONOate, a NO donor with a t\textsubscript{0.5} of <3 min, caused only a transient increase of I\textsubscript{sc}.

Mechanisms of GSNO Action: Activation of sGC and Increased cGMP Levels—Pre-treatment of Calu-3 cells with the sGC inhibitor ODQ (5 μM twice a day for 3 days) totally prevented the GSNO-induced increase in I\textsubscript{sc}.
(Fig. 7 and Table 3), indicating that sGC was involved in the GSNO-induced increase of $I_{sc}$. Subsequent addition of forskolin or cpt-cAMP into the apical compartment of an Ussing chamber containing ODQ-treated cells resulted in a significant increase of $I_{sc}$ (Fig. 7), indicating that the cells remained viable and able to respond to agents that increase cellular cAMP content. Additional evidence for the involvement of sGC is rendered by the fact that BAY 41-2272, a non-NO-based direct stimulator of sGC (24), also increased $I_{sc}$ in a dose-response fashion (Fig. 7). Steady-state concentrations of cGMP levels were increased considerably, both at 3 and 30 min post GSNO addition (Fig. 8). In contrast, cAMP values remained at baseline levels (Fig. 5E). Addition of oxy-Mb at 30 min post GSNO decreased GMP to control levels within 2 min, corresponding to the decrease of $I_{sc}$ seen in Fig. 6.

In subsequent experiments, we investigated the possibility that the observed increases of $I_{sc}$ secondary to addition of GSNO were cGMP-mediated. Addition of cpt-cGMP into the apical compartment

**TABLE 2**

GSNO (10 μM) increases Cl⁻ secretion across basolaterally permeabilized monolayers

Calu-3 cells were grown on confluent monolayers and mounted in Ussing chambers containing 5 mM in the apical and 125 mM Cl⁻ in the basolateral compartment. Amphotericin B (10 μM) was added into the basolateral compartment followed 10–20 min later by ouabain (1 mM). Once $I_{sc}$ and $Rt$ had stabilized, GSNO (10 μM) was added into the apical compartment, and changes in $I_{sc}$ and $Rt$ (data not shown) were recorded. ΔGSNO_plate. = difference in $I_{sc}$ plateau values before and after addition of GSNO; Δinh. = difference in $I_{sc}$ plateau values before and after addition of the glibenclamide, CFTRinh–172, or oxy-Mb as noted (in the presence of GSNO). Values are mean $I_{sc} ± 1$ S.E.; n = number of monolayers.

| Baseline  | Peak  | Plateau | Administration of | Δ_plate. | Δ_inh. | n |
|----------|-------|---------|------------------|----------|--------|---|
|          |       |         | glib. CFTRinh–172 oxy-Mb |          |        |   |
| $-2.8 ± 2.1$ | $28 ± 4^a$ | $4.2 ± 3.3^a$ | $-9.2 ± 2.1$ | $7.0 ± 2.6$ | $134 ± 2.7$ | 9 |
| $-3.2 ± 1.0$ | $17.7 ± 6^a$ | $3.7 ± 2.3^a$ | $-1.6 ± 2.1$ | $6.9 ± 2.8$ | $53 ± 1.6$ | 4 |
| $-6.0 ± 1.4$ | $19 ± 9^a$ | $2.6 ± 4.7^a$ | $-6.3 ± 2.8$ | $8.4 ± 4.5$ | $8.9 ± 3.5$ | 4 |

*p < 0.05 compared to each corresponding baseline value (analysis of variance followed by modified t-test).
TABLE 3

Pretreatment of confluent Calu-3 monolayers with ODQ or Oxy-Mb prevented the GSNO (10 μM) increase of Cl⁻ secretion

| Treatment       | Baseline | Peak | Plateau | ΔPeak | ΔPlateau | n  |
|-----------------|----------|------|---------|-------|----------|----|
| Vehicle         | 9.1 ± 1.1| 19 ± 2*| 14.4 ± 2*| 10 ± 2| 5.3 ± 0.8| 11 |
| ODQ (5 μM)      | 7.3 ± 2.0| 8.0 ± 2.3| 8.7 ± 2.2| 0.7 ± 0.6| 1.4 ± 0.5*| 9  |
| Vehicle         | 8.7 ± 2.2| 17.4 ± 3.3*| 16.4 ± 3.3*| 8.7 ± 3.1| 7.7 ± 3.1| 10 |
| Oxy-Mb (0.3 mM)| 8.9 ± 2.0| 9.1 ± 2.0| 9.6 ± 2.1| 0.3 ± 0.4*| 1.6 ± 0.2*| 11 |

* p < 0.05 compared to each corresponding baseline value (analysis of variance followed by modified t-test).

\(\Delta p < 0.05\) compared to the corresponding vehicle value (\(t\)-test).

increased \(I_{sc}\) (Fig. 9). No additional increase of \(I_{sc}\) was noted after subsequent addition of GSNO and/or forskolin, indicating that maximum stimulation of CFTR was achieved.

To assess a putative mechanism responsible for the activation of CFTR, we added two CFTR-specific phosphatases (PP2A1 and PP2A2, 2 milli-

units/ml each) into the apical compartment of Ussing chambers containing Calu-3 cells, following the addition of GSNO. As seen in Fig. 10 PP2A1 and PP2A2, two phosphatases known to act on CFTR but not equivalent vehicle...
solutions, decreased the GSNO-induced $I_{sc}$ shortly after being added into the apical compartment. Subsequent addition of GSNO increased the $I_{sc}$, indicating that the action of phosphatases was not due to modulation of NO release from GSNO (data not shown). These findings are consistent with NO-induced phosphorylation of CFTR and concomitant increase of its activity.

**Activation of CFTR via NO-independent Mechanisms**—Previous studies have shown that S-nitrocysteine may cross the plasma membranes via amino acid transport system L and trans-nitrosate intracellular proteins without the intermediate formation of NO (20). We thus added cysteine (50 μM) into the apical compartment of GSNO. However, addition of cysteine after GSNO did not increase $I_{sc}$, perhaps because $I_{sc}$ was already maximally stimulated by GSNO. However, in this case, addition of 0.3 mM oxy-Mb, failed to decrease the GSNO-induced $I_{sc}$ (Fig. 11).

**DISCUSSION**

The main findings of this study are that apical GSNO, in concentrations likely to be present in the normal and injured airway and alveolar fluids (1–10 μM) (10), increases Cl$^{-}$ secretion (as assessed by changes of $I_{sc}$) across confluent monolayers of Calu-3 cells, by both cGMP-dependent and -independent mechanisms. Our data indicate that GSNO

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**FIGURE 7.** Upper panel, pre-treatment of Calu-3 cells with ODQ prevents the GSNO increase of $I_{sc}$. Monolayers of Calu-3 cells were pre-treated with the sGC inhibitor ODQ (5 μM) for 72 h as described under “Material and Methods.” ODQ (5 μM) was also added in both compartments of the Ussing chamber, at least 60 min prior to the addition of GSNO. cpt-CAMP (0.05 mM) was also added into the apical compartment approximately 1 h after GSNO. Results of a typical experiment are shown. See Table 3 for mean values. Middle panel, the sGC activator BAY 41-2272 stimulates $I_{sc}$ across Calu-3 cells. Increasing concentrations of BAY 41-2272 (0.01–10 μM) or an equivalent amount of vehicle were added into the apical compartment till new steady-state values of $I_{sc}$ were achieved. Gilben-clamide (0.3 mM) was also added apically at the indicated time. Results of a typical experiment are shown. Lower panel, mean values of $\Delta I_{sc} = \pm$ S.E. of (calculated as the difference in $I_{sc}$ before and after applications of BAY or vehicle). n = 12 for BAY and 7 for Veh. (Me$_2$SO); *, $p < 0.05$ compared with the corresponding vehicle control (t-tests).

**FIGURE 8.** GSNO increases cGMP but not cAMP levels in Calu-3 cells. Upper panel, GSNO (10 μM) and cysteine (50 μM) or an equivalent volume of vehicle was added into the apical compartments of Ussing chambers containing confluent monolayers of Calu-3 cells. Monolayers were removed either at 3 or 30 min later, and cGMP content was measured as described in the text. In an additional set of experiments, cGMP levels were measured in monolayers in which oxy-Mb (0.3 mM) was added into the apical compartment 30 min post-GSNO (10 μM) and GSNO + cysteine (Cyst.) (50 μM). Values are means ± 1 S.E. The numbers in parentheses above each bar indicate number of monolayers for each group; *, $p < 0.05$ as compared with the corresponding vehicle (Veh.) control value. Lower panel, GSNO (10 μM) or an equivalent volume of vehicle was added into the apical compartment. Filters were removed at either 3 or 30 min later, and cAMP content was measured as described in the text. Values are means ± 1 S.E.; numbers in parentheses above each bar indicate number of monolayers for each group.


Releases sufficient NO, to activate sGC, which increases cGMP and possibly phosphorylates either CFTR or other chaperon proteins important in its function as a Cl⁻ channel. Indeed, the time course of \( I_{sc} \) following apical addition of GSNO paralleled the NO formation profiles in the Ussing chamber as measured by an NO electrode (an immediate transient followed by a sustained increase). Also, addition of the short acting NO-donor diethylamine NONOate resulted in only a transient increase followed by a sustained increase. Also, addition of the short acting NO-donor diethylamine NONOate resulted in only a transient increase followed by a sustained increase. Also, addition of the short acting NO-donor diethylamine NONOate resulted in only a transient increase followed by a sustained increase. Also, addition of the short acting NO-donor diethylamine NONOate resulted in only a transient increase followed by a sustained increase.

Experiments across basolaterally permeabilized monolayers show that GSNO added into the apical compartment activates apical pathways of Cl⁻ secretion. The fact that the induced \( I_{sc} \) was inhibited both by

glibenclamide (a \( K_{ATP} \) and CFTR blocker) and CFTR-inh-172, a cell-permeable thiazolidinone compound that acts as a potent, reversible, rapid, and voltage-independent inhibitor of CFTR (25), strongly suggests activation of CFTR by GSNO.

Calu-3 cells also contain Ca²⁺-activated Cl⁻ channels; however, addition of the Ca²⁺ ionophore ionomycin into the apical compartment of Ussing chambers containing Calu-3 cells caused only a transient stimulation of \( I_{sc} \) lasting no more than a few minutes (data not shown); thus activation of Ca²⁺-activated Cl⁻ channels, even if it occurred, cannot account for the sustained GSNO-induced increase of \( I_{sc} \) observed in these studies. Also, additional studies have reported that other NO donors such as nitroprusside or N-nitroso-N-acetylpenicillamine cause a dose-dependent decrease of both apical and basolateral Na⁺-K⁺-2Cl⁻ co-transporters in a renal epithelial cell line (26). Thus, it is unlikely that the observed increase of \( I_{sc} \) by GSNO was due to activation of basolateral Cl⁻ transporters.

It is widely accepted that phosphorylation of the regulatory domain of CFTR by protein kinase A, followed by binding and hydrolysis of ATP at its nucleotide binding domain, is the main event leading to its activation (27). Agents that increase cAMP increase vectorial Cl⁻ secretion across both native secretory epithelial (6, 9, 28, 29) and heterologous expressing CFTR cells (30). However, contrary to what has been reported in endothelial cells (31), GSNO did not increase cAMP levels in Calu-3

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**FIGURE 9.** Forskolin and cpt-cGMP increase \( I_{sc} \) across Calu-3 cells. Forskolin (10 \( \mu \)M, middle panel); added into the apical compartment, increased \( I_{sc} \). Subsequent addition of either GSNO or forskolin (which increases cAMP concentration) did not increase \( I_{sc} \) further. Results of a typical experiment are shown. Lower panel, mean values ± 1 S.E. Mean steady state values of calu-3 \( I_{sc} \) ± 1 S.E. following addition of the following compounds in the apical compartments of Ussing chambers; \( n \) number of monolayers in each group: 1, vehicle (n = 20); 2, GSNO (10 \( \mu \)M, n = 5; 3, forskolin (10 \( \mu \)M, n = 7); 4, cpt-cGMP (1 \( \mu \)M, n = 8); 5, GSNO (10 \( \mu \)M)+forskolin (10 \( \mu \)M, n = 4); 6, GSNO (10 \( \mu \)M)+ cpt-cGMP (1 \( \mu \)M, n = 8). *p < 0.05 as compared with vehicle (one way analysis of variance). All values are significantly different (p < 0.5 compared with control).

**FIGURE 10.** The phosphatases PP2A1 and PP2A2 reverse the GSNO-induced increase of \( I_{sc} \) in Calu-3 cells. Upper panel, following addition of GSNO (10 \( \mu \)M) into the apical compartment and achievement of a new steady-state \( I_{sc} \) vehicle solution (prepared according to the manufacture's specifications) or 2 milliunits/ml each of protein phosphatase type 2A1 and type 2A2 (abbreviated as PP2A) were added into the apical compartments. Results of a typical experiment are shown. Lower panel, mean values ± 1 S.E. for changes of \( I_{sc} \) (calculated as the difference in the plateau value after addition of GSNO minus the steady-state value after addition of PP2A1 plus PP2A2). At least 4 filters/group; *p < 0.05 compared with the corresponding vehicle.
the SIN-1 effect when added at the peak of the SIN-1 response but not when \( I_{\text{sc}} \) had returned to its baseline value. Perfusion of mouse tracheal epithelial cells with SIN-1 also increased whole cell \( \text{Cl}^- \) currents 4-fold and the open probability of CFTR-type single channel currents from 0.041 to 0.92 in a transient fashion (21). The mechanism by which ONOO\(^-\), but not NO, stimulates \( \text{Cl}^- \) secretion across mouse tracheal epithelial cells is not clear at this time.

Further insights into the cGMP-independent mechanism of GSNO activation of \( \text{Cl}^- \) secretion were provided by adding both GSNO and L-cysteine in the apical compartments of Ussing chambers containing Calu-3 cells. Recent studies (14, 20) have shown that L-cysteine provides a substrate for trans-nitrosation from GSNO, with the ensuing product S-nitrosocysteine readily being transported into cells via the amino acid transport system L (L-AT). Zhang and Hogg (20) showed an almost 50-fold increase in S-nitrosothiol content of RAW 264.7 cells incubated with GSNO and L-cysteine and significant inhibition of this process by BCH and L-leucine, inhibitors of the L-AT transporter. CFTR contains 18 intracellular cysteines (16) that may become nitrosated by these species. Post-translational modification (oxidation or nitrosation) of \( \Delta F_{508}\text{CFTR} \) by GSNO has been associated with increased maturation (15). In our experiments, L-cysteine also caused a transient release of NO from GSNO as measured by an NO\(^-\) electrode in the presence of cells, consistent with thiol-mediated 1-electron reduction of GSNO to NO, as has been previously observed with a number of nitrosothiols, including S-nitroso-N-acetylpenicillamine (14, 36). However, no additional increase of \( I_{\text{sc}} \) was noted, most likely because CFTR was already maximally stimulated by NO.

Oxy-Mb, even in high concentrations (0.4 mM), failed to modulate the GSNO plus cysteine-induced increase of \( I_{\text{sc}} \), suggesting that either formation of NO within Calu-3 cells prevents scavenging by oxy-Mb or intracellular S-nitrosothiols are stimulating \( \text{Cl}^- \) currents independent of NO formation. On the basis that NO is relatively diffusible, it is unlikely that NO formation within cells can preclude scavenging by an efficient trap present outside the cells, a fact consistent with the hypertensive effects of cell-free hemoglobin (37, 38). More importantly, in the presence of L-cysteine, GSNO-stimulated cGMP formation declined to baseline upon addition of oxy-Mb. The fact that this was not associated with inhibition of \( I_{\text{sc}} \) strongly suggests that, in the presence of L-cysteine, GSNO stimulates \( I_{\text{sc}} \) independent of NO formation, and sGC stimulation likely occurs via intracellular S-nitrosothiol-dependent effects that could include direct S-nitrosation or S-glutathioylation of critical protein thiols. Ongoing experiments are evaluating the potential targets of S-nitrosothiols that could modulate \( I_{\text{sc}} \) in Calu-3 cells.

As mentioned above, post-translational modifications of both wild-type and mutant CFTR has been reported in a number of studies. Most recently Wang et al. (16) reported that oxidized forms of glutathione (such as GSNO) decreased CFTR currents in membrane-excised patches from Baby hamster kidney cells, stably transfected with CFTR (BHK-CFTR) within a few minutes by glutathionylating Cys-1344. Based on the fact that S-nitroso-N-acetylpenicillamine did not decrease CFTR function, they concluded that the effects of GSNO were not due to the release of NO. However, NO release by S-nitroso-N-acetylpenicillamine involves trans-nitrosation reactions requiring the presence of significant levels of cysteine (36). Contrary to their observations, in our experiments oxidized glutathione (10 \( \mu \)M) did not alter \( \text{Cl}^- \) secretion across Calu-3 cells. Zaman and Palmer (15, 39) reported that exposure of a variety of cells to GSNO causes an increase in mature CFTR after 4 h. This change was prevented by dithiothreitol and thus was attributed to oxidative modification of CFTR. In our studies we found no change in surface expression of CFTR of Calu-3 exposed to 50 \( \mu \)M GSNO for up to

cells; furthermore, ODQ, a well known specific inhibitor of sGC, prevented the GSNO increase of \( I_{\text{sc}} \) across Calu-3 cells. These findings indicate that activation of sGC/cGMP were responsible for the noted effects in our experiments.

In previous studies, Berger et al. (30) showed the type Ic cGMP dependent protein kinase phosphorylated CFTR in vitro, and PP2A dephosphorylated CFTR. However, in contrast to protein kinase A, phosphorylation of CFTR by cGKI failed to activate \( \text{Cl}^- \) secretion across human airway cells. Another membrane isoform of cGK (cGKII) has been isolated from intestinal epithelium by de Jong et al. (32), which is not present in airway epithelial cells. In subsequent studies it was shown that, although both isoforms phosphorylate CFTR, only cGKII activated CFTR channels in membrane patches and \( \text{Cl}^- \) currents across cells patched in the whole cell mode (33). Furthermore, transfection of cells expressing CFTR with cGKII, but not cGKI, allowed cGMP-activation of CFTR (34). Thus it is likely that Calu-3 but not mouse tracheal epithelial cells express cGKII allowing phosphorylation and activation of CFTR (or critical chaperon proteins) by agents that increase cGMP.

We also failed to see a significant increase of \( \text{Cl}^- \) secretion across confluent monolayers of mouse tracheal epithelial cells by GSNO and cGMP. On the other hand, addition of 3-morpholinosydnonimine (SIN-1, 1 mM), a peroxynitrite (ONOO\(^-\)) donor, into the apical compartment of amiloride-treated monolayers of mouse tracheal epithelial cells resulted in a transient increase of \( I_{\text{sc}} \). Forskolin (10 \( \mu \)M) augmented
6 h (35). However, prolonged exposure of Calu-3 cells to DETA NONOate (100 µM for 48–72 h) resulted in nitration of CFTR, decreased apical CFTR levels, and cAMP-regulated Cl channel current (9).

In summary, our results provide direct evidence that GSNO stimulates Cl − secretion across Calu-3 cells by both cGMP-dependent and -independent mechanisms. Nitratosothiolates may be contributing to basal activation of CFTR in vivo.

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