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**Carbon monoxide-releasing molecules inhibit the cystic fibrosis transmembrane
conductance regulator Cl⁻ channel**

Mayuree Rodrat,^{1,2,3*} Walailak Jantarajit,^{1,2,3*} Demi R. S. Ng,¹ Bartholomew S. J. Harvey,¹
Jia Liu,¹ William J. Wilkinson,⁴ Narattaphol Charoenphandhu,^{2,3,5,6} and David N. Sheppard¹

¹ School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, UK,

² Center of Calcium and Bone Research (COCAB), Faculty of Science, Mahidol University,
Bangkok, Thailand,

³ Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand,

⁴ School of Biosciences, Cardiff University, Cardiff, UK,

⁵ Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand

and

⁶ The Academy of Science, The Royal Society of Thailand, Dusit, Bangkok, Thailand

Running Title: Carbon monoxide-releasing molecules inhibit CFTR

*Author contributions: MR and WJ are co-first author

26 Address Correspondence to: D.N. Sheppard, Ph.D.
27 University of Bristol
28 School of Physiology, Pharmacology and Neuroscience
29 Biomedical Sciences Building
30 University Walk
31 Bristol BS8 1TD
32 United Kingdom
33 Tel: +44 117 331 2290
34 E-mail: D.N.Sheppard@bristol.ac.uk
35

36 ABSTRACT

37 The gasotransmitter carbon monoxide (CO) regulates fluid and electrolyte movements
38 across epithelial tissues. However, its action on anion channels is incompletely understood.
39 Here, we investigate the direct action of CO on the cystic fibrosis transmembrane
40 conductance regulator (CFTR) by applying CO-releasing molecules (CORMs) to the
41 intracellular side of excised inside-out membrane patches from cells heterologously
42 expressing wild-type human CFTR. Addition of increasing concentrations of
43 tricarbonyldichlororuthenium (II) dimer (CORM-2) (1 – 300 μ M) inhibited CFTR channel
44 activity, whereas the control RuCl_3 (100 μ M) was without effect. CORM-2 predominantly
45 inhibited CFTR by decreasing the frequency of channel openings and hence, open probability
46 (P_o). But, it also reduced current flow through open channels with very fast kinetics,
47 particularly at elevated concentrations. By contrast, the chemically distinct CO-releasing
48 molecule CORM-3 inhibited CFTR by decreasing P_o without altering current flow through
49 open channels. Neither depolarizing the membrane voltage nor raising the ATP concentration
50 on the intracellular side of the membrane affected CFTR inhibition by CORM-2.
51 Interestingly, CFTR inhibition by CORM-2, but not by CFTR_{inh}-172, was prevented by prior
52 enhancement of channel activity by the clinically-approved CFTR potentiator ivacaftor.
53 Similarly, when added after CORM-2, ivacaftor completely relieved CFTR inhibition. In
54 conclusion, CORM-2 has complex effects on wild-type human CFTR consistent with
55 allosteric inhibition and open-channel blockade. Inhibition of CFTR by CO-releasing
56 molecules suggests that CO regulates CFTR activity and that the gasotransmitter has tissue-
57 specific effects on epithelial ion transport. The action of ivacaftor on CFTR Cl^- channels
58 inhibited by CO potentially expands the drug's clinical utility.

59

60 Keywords: CFTR chloride ion channel / channel inhibition / carbon monoxide-releasing
61 molecule 2 (CORM-2) / CFTR potentiation / ivacaftor (VX-770)

62 INTRODUCTION

63 Widely expressed in epithelial cells lining ducts and tubes throughout the body, the
64 cystic fibrosis transmembrane conductance regulator (CFTR) (56) plays a pivotal role in
65 epithelia as testified by its dysfunction in the common life-shortening genetic disease cystic
66 fibrosis (CF) (54, 58, 67). Assembled from two membrane-spanning domains (MSDs), two
67 nucleotide-binding domains (NBDs) and a unique regulatory domain (RD) (56), CFTR
68 (ABCC7) is an anion channel with the architecture of an ATP-binding cassette (ABC)
69 transporter (22, 23, 36, 90). Once the RD is phosphorylated by protein kinase A (PKA) (45),
70 CFTR gating is controlled by cycles of ATP binding and hydrolysis at the NBDs driving
71 conformational changes in the MSDs, which open and close the channel pore (19, 23).

72
73 CFTR-mediated fluid and electrolyte movement across epithelial tissues involves the
74 coordinated action of ion channels, transporters and pumps located in the apical and
75 basolateral membranes of individual epithelial cells tightly regulated by a network of
76 intracellular signaling pathways (18). It is now recognized that nitric oxide (NO), hydrogen
77 sulfide (H₂S) and carbon monoxide (CO) act as endogenous gasotransmitters regulating the
78 activity of ion channels and transporters in different tissues, including epithelia (1, 50, 85).
79 Previous work demonstrates that NO and H₂S stimulate CFTR activity by modulating the
80 phosphorylation status of CFTR. For example, in intestinal epithelial cells, NO robustly
81 increases cGMP production (86). In turn, cGMP activates the membrane-localized type II
82 cGMP-dependent protein kinase, leading to opening of the CFTR Cl⁻ channel (17, 71). By
83 contrast, in *Xenopus* oocytes heterologously expressing CFTR, H₂S inhibits cAMP
84 breakdown by phosphodiesterases to promote CFTR activation by PKA (49). Less is known
85 about the regulation of CFTR by CO. With the Ussing chamber technique, two studies
86 demonstrated that the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer

87 (CORM-2) stimulated Cl^- secretion by intestinal epithelia, in part, by activating anion
88 channels located in the apical membrane (66, 70). Based on these and other data (77, 78),
89 Wang (79) speculated that CO stimulates CFTR by relieving CFTR inhibition by Fe^{3+} .

90

91 In this study, we investigated the direct action of CO on the CFTR Cl^- channel. Using
92 CORM-2 and excised inside-out membrane patches from cells heterologously expressing
93 wild-type human CFTR, we studied the effects of CO on the single-channel behavior of
94 CFTR. We discovered that CORM-2 has complex effects on CFTR, impeding channel gating
95 and obstructing current flow through open channels with characteristics similar to those of
96 allosteric inhibitors and open-channel blockers of CFTR. Of note, the action of CORM-2 on
97 CFTR was abolished by ivacaftor, a CFTR-targeting drug used clinically to treat CF (53, 72).
98 These results inform studies of the physiological role of CFTR and the therapeutic utility of
99 ivacaftor.

100

101 **METHODS**

102 **Cells and cell culture**

103 For this study, we used mouse mammary epithelial (C127) cells stably expressing
104 wild-type human CFTR (39). These cells were a generous gift of C. R. O’Riordan (Sanofi
105 Genzyme). They were cultured and used as described previously (62).

106

107 **Patch-clamp experiments**

108 CFTR Cl^- channels were recorded in excised inside-out membrane patches using
109 Axopatch 200A and 200B patch-clamp amplifiers and pCLAMP software (version 10.4) (all
110 from Molecular Devices, San Jose, CA) as described previously (62). Unless otherwise
111 indicated, the pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine

(NMDG), 140 aspartic acid, 5 CaCl₂, 2 MgSO₄ and 10 N-tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), adjusted to pH 7.3 with Tris ([Cl⁻], 10 mM). The bath (intracellular) solution contained (mM): 140 NMDG, 3 MgCl₂, 1 CsEGTA and 10 TES adjusted to pH 7.3 with HCl ([Cl⁻], 147 mM; free [Ca²⁺], < 10⁻⁸ M). Using a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL), the temperature of the bath solution was maintained at 37 °C.

After excision of inside-out membrane patches, we clamped voltage at -50 mV and added the catalytic subunit of PKA (75 nM) and ATP (1 mM) to the intracellular solution within 5 minutes of membrane patch excision to activate CFTR Cl⁻ channels. To test the effects of CORM-2 and other small molecules on the single-channel behavior of wild-type CFTR, we reduced the ATP concentration to 0.3 mM before adding compounds to the intracellular solution in the continuous presence of ATP (0.3 mM) and PKA (75 nM). Once channel activity stabilized following compound addition, we acquired 3 – 8 minutes of single-channel data. Because of the difficulty of removing ivacaftor from the recording chamber (84), specific interventions were compared with the pre-intervention control period made with the same concentration of ATP and PKA, but without test small molecules. To minimize channel rundown, PKA and ATP were added to all intracellular solutions. To investigate the voltage-dependence of CFTR inhibition, we used symmetrical Cl⁻-rich solutions and stepped voltage from 0 mV to either -50 mV for 60 s or +50 mV for 30 s. We chose 30 s steps at +50 mV because this time interval was long enough to acquire sufficient transitions to quantify open probability (*P*_o), but short enough to prevent seal breakdown and loss of the excised inside-out membrane patch. We did not step voltage to voltages beyond ±50 mV because of the weak inward rectification of CFTR Cl⁻ currents at large positive voltage (7).

In this study, we used excised inside-out membrane patches containing ≤ 5 active channels. To determine channel number, we used the maximum number of simultaneous channel openings observed during an experiment (9). To minimize errors, we used experimental conditions that robustly potentiate channel activity and verified that recordings were of sufficient length to determine the correct number of channels (74).

Single-channel currents were acquired directly to computer hard disc after filtering at a corner frequency (f_c) of 500 Hz using an eight-pole Bessel filter (model F-900C/9L8L, Frequency Devices Inc., Ottawa, IL) and digitized at a sampling rate of 5 kHz using a DigiData 1440A interface (Molecular Devices) and pCLAMP software (version 10.4). For Figure 7, data were additionally digitally filtered at 100 Hz prior to analysis. To measure single-channel current amplitude (i), Gaussian distributions were fit to current amplitude histograms. For P_o measurements, lists of open- and closed-times were generated using a half-amplitude crossing criterion for event detection and dwell time histograms constructed as previously described (62); transitions < 1 ms were excluded from the analysis (eight-pole Bessel filter rise time (T_{10-90}) ~ 0.73 ms at $f_c = 500$ Hz) with the exception of Figure 7, where transitions < 4 ms were excluded. Histograms were fitted with one or more component exponential functions using the maximum likelihood method. To determine which component function fitted best, the log-likelihood ratio test was used and considered statistically significant at a value of 2.0 or greater (87). For burst analyses, we used a t_c (the time that separates interburst closures from intraburst closures) determined from closed time histograms [control, $t_c = 18.8 \pm 5.2$ ms ($n = 5$); ivacaftor, $t_c = 10.8 \pm 4.4$ ms ($n = 5$); ivacaftor + CORM-2, $t_c = 11.1 \pm 1.6$ ms ($n = 5$); ivacaftor + CORM-2 + CFTR_{inh}-172, $t_c = 24.1 \pm 1.7$ ms ($n = 4$)] (9). The mean interburst interval (T_{IBI}) was calculated using the equation (9):

$$P_o = T_b / (T_{\text{MBD}} + T_{\text{IBI}}), \quad (\text{Eq. 1})$$

where, T_b = (mean burst duration) x (open probability within a burst). Mean burst duration (T_{MBD}) and open probability within a burst ($P_{o(\text{burst})}$) were determined directly from experimental data using pCLAMP software. Only membrane patches that contained a single active channel were used for burst and kinetic analyses. For the purpose of illustration, single-channel records were filtered at 500 Hz and digitized at 5 kHz before file size compression by 5-fold data reduction.

Reagents

PKA purified from bovine heart was purchased from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). Ivacaftor was purchased from Selleck Chemicals (Strattech Scientific Ltd., Newmarket, UK), while all other chemicals, including CORM-2, CORM-3, RuCl_3 and CFTR_{inh}-172 were supplied by Sigma-Aldrich Ltd. (now Merck Life Science UK Ltd.) (Gillingham, UK).

Stock solutions of ATP were prepared in intracellular solution directly before each experiment. Except for CORM-3, which was dissolved in deionized water, all other stock solutions were solubilized in DMSO before storage at -20°C . Immediately before use, stock solutions were diluted to final concentrations with intracellular solution and, where necessary, the pH of the intracellular solution was readjusted to pH 7.3 to avoid pH-dependent changes in CFTR function (10). Precautions against light-sensitive reactions were observed when using test small molecules. DMSO was without effect on the single-channel behavior of CFTR (62). On completion of experiments, the recording chamber was thoroughly cleaned before re-use (84).

Statistics

Data recording and analyses were randomized, but not blinded. Results are expressed as means $\pm$ SEM of n observations, but some group sizes were unequal due to technical difficulties with the acquisition of single-channel data. All data were tested for normal distribution using a Shapiro-Wilk normality test. To test for differences between two groups of data acquired within the same experiment, we used Student's paired t -test. To test for differences between multiple groups of data, we used one-way, repeated measures analysis of variance (ANOVA) followed by either Dunnett's or Tukey multiple comparison test when a statistically significant difference was observed. Tests were performed using SigmaPlotTM (version 13.0, Systat Software Inc., San Jose, CA) with the exception that concentration-response relationships were analyzed using Prism (version 5.0, GraphPad Software, San Diego, CA). Differences were considered statistically significant when $P < 0.05$. In patch-clamp experiments, n represents the number of individual membrane patches obtained from different cells. To avoid pseudo-replication, all experiments were repeated at different times.

Data accessibility statement

Data are available at the University of Bristol data repository, data.bris, at <https://doi.org/10.5523/bris.12vo5en267fwo2x22o6afexii1>.

RESULTS

Carbon monoxide-releasing molecules inhibit wild-type CFTR

In this study, we investigated the direct action of CORM-2 on wild-type human CFTR using cell-free membrane patches from cells heterologously expressing CFTR. Following CFTR activation by PKA-dependent phosphorylation, we acutely added increasing

concentrations of CORM-2 to the intracellular solution in the continuous presence of PKA (75 nM) and ATP (0.3 mM). Figure 1 demonstrates that CORM-2 inhibited the activity of wild-type CFTR in two ways. First, CORM-2 altered the gating pattern of CFTR. The gating behavior of wild-type human CFTR is characterized by bursts of channel openings interrupted by brief, flickery closures, separated by longer closures between bursts (Fig. 1A). Addition of CORM-2 to the intracellular solution predominantly decreased the frequency of channel openings, but at higher concentrations, it also decreased markedly the duration of channel openings (Fig. 1). Second, CORM-2 altered current flow through open channels. Figure 1A and B demonstrate that elevated concentrations of CORM-2 decreased notably the single-channel current amplitude (i) of wild-type CFTR. To quantify channel inhibition by CORM-2, we measured i and P_o . Figure 1C and D show the effects of raising the CORM-2 concentration from 1 to 300 μ M on the i and P_o of wild-type CFTR at -50 mV. For both i and P_o , the relationship between drug concentration and CFTR inhibition was well fitted by nonlinear regression functions with the effect of CORM-2 on P_o stronger than its action on i (i : $IC_{50} = 46.8 \pm 1.7 \mu$ M; P_o : $IC_{50} = 31.0 \pm 1.6 \mu$ M; $n = 6 - 17$) (Fig. 1C and D). Channel inhibition by CORM-2 was partially reversible ($n = 3$; data not shown). Thus, CORM-2 has complex effects on the CFTR Cl^- channel.

The chemical structure of the metal carbonyl complex CORM-2 (tricarbonyldichlororuthenium (II) dimer) is distinct from those of small molecules that inhibit the CFTR Cl^- channel (33, 41). As controls, we tested the effects on wild-type human CFTR of $RuCl_3$ and tricarbonylchloro(glycinato)ruthenium (II) (CORM-3), a CO-releasing molecule with a distinct chemical structure, which like CORM-2 rapidly releases CO (11, 41, 43). Figure 2 demonstrates that acute addition of $RuCl_3$ (100 μ M) to the intracellular solution bathing excised inside-out membrane patches was without effect on the CFTR Cl^- channel.

Neither current flow through open channels nor channel gating and hence, P_o were affected by exposure to RuCl_3 (100 μM) (Fig. 2). By contrast, Figure 3 demonstrates that acute addition of CORM-3 (30 – 300 μM) to the intracellular solution inhibited wild-type CFTR. Comparison of the data in Figures 1 and 3 reveals two important differences between the two CO-releasing molecules. First, unlike CORM-2, CORM-3 was without effect on i ; it only inhibited P_o by delaying channel opening (Figs. 1 and 3). Second, CORM-3 inhibited wild-type CFTR less potently than CORM-2 (Figs. 1D and 3D). It was also less efficacious than the widely used CFTR inhibitor $\text{CFTR}_{\text{inh}}-172$, an allosteric inhibitor of channel gating (31, 38) (Fig. 3). Taken together, the data demonstrate that acute addition of CO-releasing molecules to the intracellular solution directly inhibits the CFTR Cl^- channel. They also reveal that CORM-2 and CORM-3 impede channel opening and that CORM-2 additionally obstructs current flow through open channels.

Mechanistic studies of CORM-2 inhibition of wild-type CFTR

To understand better how CORM-2 inhibits wild-type human CFTR, we selected for study the concentration of 30 μM , close to the IC_{50} for CORM-2 inhibition of P_o . Using membrane patches containing a single active CFTR Cl^- channel, we investigated how CORM-2 decreased P_o by analyzing the kinetics of channel gating. Consistent with previous results [e.g. (87)], the open- and closed-time histograms of wild-type human CFTR were best fitted with one- and two-component exponential functions (Fig. 4A and Table 1). In this study, the two populations of channel closures described by fast ($\tau_{\text{C}2}$) and slow ($\tau_{\text{C}3}$) closed time constants, represent the brief, flickery closures that interrupt channel openings and the prolonged closures, which separate one burst of channel opening from the next.

In the presence of CORM-2 (30 μ M) open- and closed-time histograms of wild-type human CFTR were best fitted with two- and four-component exponential functions, respectively (Fig. 4B and Table 1). The new population of open times was described by a fast open-time constant (τ_{O1}), while the new populations of closed times, which represent CORM-2-induced channel inhibition, were described by very fast (τ_{C1}) and very slow (τ_{C4}) closed-time constants (Fig. 4B and Table 1). In addition, CORM-2 (30 μ M) increased the fast closed-time constant (τ_{C2}) by 58%, reduced the slow closed-time constant (τ_{C3}) by 41%, but was without effect on the slow open-time constant (τ_{O2}) (Fig. 4B and Table 1). Table 1 reveals that the share of the closed time distribution occupied by the fast (τ_{C2}) and slow (τ_{C3}) closed-time constants decreased by 38% and 14%, respectively in the presence of CORM-2 (30 μ M). As a result, the new closed-time constants τ_{C1} and τ_{C4} occupied 51% and only 1% of the closed time distribution (Table 1). Interestingly, in the presence of CORM-2 (30 μ M) the fast open time constant (τ_{O1}) dominated the open time distribution (Table 1). We interpret these data to suggest that CORM-2 has complex effects on the gating kinetics of CFTR, exhibiting similarities to the action of elevated concentrations of the CFTR potentiator genistein, which inhibits wild-type human CFTR (32).

Like genistein (32), the complex effects of CORM-2 on the CFTR Cl^- channel have characteristics in common with both open-channel blockers, which occlude the channel pore, and allosteric inhibitors that impede channel gating (23, 33). To probe the mechanism of CORM-2 inhibition of wild-type CFTR, we tested the effects of voltage and varied the intracellular ATP concentration. Many open-channel blockers occlude the intracellular vestibule of the CFTR pore, leading to voltage-dependent block (33, 35). For example, when bathed in symmetrical Cl^- -rich solutions, glibenclamide (50 μ M) potently inhibited wild-type CFTR at negative voltages, but at positive voltages inhibition was completely relieved (62).

Figure 5A shows a similar experiment using a membrane patch with two CFTR Cl⁻ channels bathed in symmetrical Cl⁻-rich solutions in the absence and presence of CORM-2 (30 μM) and summary data from six experiments. Under control conditions, the single-channel behavior of wild-type CFTR was unaffected by voltage; neither *i* nor *P*_o differed between -50 and +50 mV (Fig. 5B and C). Acute addition of CORM-2 (30 μM) to the intracellular solution potently inhibited channel gating at both voltages with the result that only brief openings of one channel were observed and *P*_o was strongly reduced at both voltages (*P*_{o(drug)}/*P*_{o(control)}: -50 mV, 29.0 ± 7.1% (*n* = 6); +50 mV, 23.0 ± 8.2% (*n* = 6); *P* = 0.16) (Fig. 5A and C). Although visual inspection of single-channel recordings indicates that some channel openings were of reduced size at both voltages in the presence of CORM-2 (30 μM), the summary data reveal no change in *i* at either voltage in the presence of CORM-2 (30 μM) (*i*_{drug}/*i*_{control}: -50 mV, 96.0 ± 3% (*n* = 6); +50 mV, 95.1 ± 3.0% (*n* = 6); *P* = 0.74) (Fig. 5A and B). We interpret these data to suggest that inhibition of wild-type CFTR by CORM-2 is voltage-independent, but its effect on current flow through open channels exhibits some dependence on the external Cl⁻ concentration based on the different effects of CORM-2 on *i* using either a Cl⁻ concentration gradient or symmetrical Cl⁻-rich solutions.

Allosteric inhibitors bind to CFTR at a site remote from the channel pore to interfere with channel gating (23, 33). Because some allosteric inhibitors [e.g. genistein (32)] interfere with ATP-dependent channel gating, we examined the effects of increasing the intracellular ATP concentration on CORM-2 inhibition of wild-type CFTR. Figure 6 shows representative single-channel recordings of wild-type CFTR using a Cl⁻ concentration gradient to demonstrate the effects of CORM-2 (30 μM) when the intracellular solution contained either 0.3 or 3 mM ATP and summary data from six to eight experiments. Consistent with previous results [e.g. (8)], elevating the ATP concentration to 3 mM noticeably increased the frequency

of channel openings and hence P_o , but had little or no effect on i (Fig. 6). However, raising the ATP concentration to 3 mM failed to relieve CFTR inhibition by CORM-2 (30 μ M) (Fig. 6). At ATP (3 mM), the CORM-2-induced reductions in i and P_o were similar to those at ATP (0.3 mM) ($i_{\text{drug}}/i_{\text{control}}$: ATP (0.3 mM), $77.9 \pm 6.8\%$ ($n = 8$); ATP (3 mM), $87.6 \pm 6.6\%$ ($n = 6$); $P = 0.28$; $P_{o(\text{drug})}/P_{o(\text{control})}$: ATP (0.3 mM), $46.5 \pm 12.2\%$ ($n = 8$); ATP (3 mM), $46.1 \pm 15.2\%$ ($n = 6$); $P = 0.8$) (Fig. 6C and D). Taken together the data demonstrate that CORM-2 does not cause voltage-dependent inhibition of wild-type CFTR nor does it inhibit channel activity by competing with ATP.

Ivacaftor prevents CFTR inhibition by CORM-2

Previous work demonstrated that the clinically-approved CFTR potentiator ivacaftor (53, 72) relieves CFTR inhibition by cigarette smoke (52). We were therefore interested to learn whether ivacaftor would modify the inhibition of wild-type CFTR by CORM-2. To test this idea, we performed two experiments, acutely adding both compounds to the intracellular solution bathing excised membrane patches from cells heterologously expressing wild-type CFTR; first we added ivacaftor (100 nM) before CORM-2 (30 μ M) and second, we reversed the order of addition of the two compounds.

Figure 7 shows the effects of sequential and cumulative addition of CORM-2 (30 μ M) and CFTR_{inh}-172 (10 μ M) on a single wild-type CFTR Cl⁻ channel potentiated by ivacaftor (100 nM). To quantify the data, we measured i , P_o and performed an analysis of bursts to determine how small molecules alter the frequency and duration of channel openings. Consistent with previous results [e.g. (83)], acute addition of ivacaftor (100 nM) to the intracellular solution potentiated CFTR channel gating by increasing the frequency and duration of channel openings, leading to an 66% increase in P_o , but no change in i (Fig. 7).

Figure 7 demonstrates that once wild-type CFTR was potentiated by ivacaftor (100 nM), CORM-2 (30 μ M) was without effect on current flow through open channels and had little or no effect on the pattern of channel gating. Neither i , mean burst duration (MBD) nor interburst interval (IBI) were altered by the small molecule, while P_o was reduced only 18% compared to that potentiated by ivacaftor (100 nM) (Fig. 7). By contrast, subsequent addition of CFTR_{inh}-172 (10 μ M) had a marked effect on channel gating, increasing IBI 2,035% and reducing MBD 52% without altering i (Fig. 7). These effects of CFTR_{inh}-172 (10 μ M) after CORM-2 (30 μ M) closely resemble its action on wild-type CFTR potentiated by ivacaftor (100 nM) in the absence of CORM-2 (25).

Finally, we tested whether ivacaftor would relieve the inhibition of wild-type CFTR by CORM-2. Figure 8 shows that CORM-2 (30 μ M) reduced i 10% and P_o 57%. However, subsequent acute addition of ivacaftor (100 nM) to the intracellular solution restored values of i and P_o to control levels before CORM-2 (30 μ M) exposure (Fig. 8). Like its effect after ivacaftor and CORM-2 (Fig. 7), the ensuing addition of CFTR_{inh}-172 (10 μ M) to the intracellular solution strongly inhibited the P_o of wild-type CFTR, without altering i (Fig. 8). Thus, like its effects on CFTR inhibition by cigarette smoke (52), ivacaftor prevents wild-type CFTR inhibition by CORM-2.

DISCUSSION

This study investigated the direct action of CORM-2 on wild-type human CFTR using the patch-clamp technique. Our data demonstrate that CORM-2 impedes channel gating and obstructs current flow through CFTR. However, its action is abolished by the clinically-approved CFTR potentiator ivacaftor.

The present results contrast with previous studies of CORM-2 on transepithelial ion transport with the Ussing chamber technique, which demonstrated that the small molecule activated anion channels in the apical membrane of cultured human intestinal (Caco-2) and rat colonic epithelia (66, 70). Using similar concentrations of CORM-2, we only observed inhibition of the CFTR Cl^- channel. Moreover, we found no evidence for dual effects, unlike some CFTR modulators, which either potentiate or inhibit channel activity depending on the experimental conditions employed [e.g. genistein (32, 76); NPPB (34, 82); phloxadine B (8)]. Species differences in CFTR pharmacology [e.g. (4, 65)] is an unlikely explanation for the results obtained. By contrast, variation in the complement of transport proteins, signaling molecules and CFTR-interacting proteins in the different cells used to study CFTR [Caco-2 cells (70); rat distal colon (66); C127 cells (present study)] is a potential explanation. Similarly, using cell-free membrane patches, cytosolic regulatory factors might be lost, whereas in polarized intestinal epithelia they would be retained (66, 70) and the present study).

However, the most likely explanation for the different results is the action of CO on distinct types of apical membrane anion channels. Under the experimental conditions used by Uc et al. (70) and Steidle and Diener (66), it is feasible that the inhibitors DIDS, glibenclamide and NPPB might have decreased short-circuit current (I_{sc}) by targeting other anion channels, not CFTR. Consistent with this idea, in rat epididymal epithelia, the NPPB- and DIDS-sensitive CORM-2-induced increase in I_{sc} was robustly inhibited by chelation of intracellular Ca^{2+} with BAPTA-AM, whereas the CFTR inhibitor CFTR_{inh}-172 had little effect (48). These data suggest that in rat epididymal epithelia CORM-2 increases I_{sc} by targeting the Ca^{2+} -activated Cl^- channel TMEM16A (47). Interestingly, CORM-2 and CORM-3 inhibited cAMP signaling and cAMP-stimulated I_{sc} in cultured human bronchial

epithelia (16HBE14o⁺) (89), while hypercapnia had similar effects on primary cultures of human airway epithelia (69).

We found that two CO-releasing molecules, CORM-2 and CORM-3, acutely inhibited the CFTR Cl⁻ channel in excised inside-out membrane patches, whereas RuCl₃ was without effect. Because the biological activity of CORM-2 and CORM-3 is directly related to CO (11, 42), the simplest interpretation of the data is that CO inhibits CFTR. However, the diverse chemical structures of CFTR inhibitors (33) cautions that the CO-releasing molecules, themselves, not CO, might mediate the observed effects. Interestingly, CORM-2, a lipid soluble CO-releasing molecule (41), inhibited CFTR with greater potency and efficacy than CORM-3, a water-soluble compound (11). We interpret these results to suggest that the action of CO-releasing molecules involves the lipid bilayer or the MSDs of CFTR, which interact with it. Consistent with this idea, ivacaftor, prevented CFTR inhibition by CORM-2, but not CFTR_{inh}-172. Interestingly, ivacaftor binds CFTR at the MSD-lipid interface with residues from the fourth transmembrane segment (M4), M5 and the unstructured region of M8 contributing to this potentiator-binding pocket (37, 88). Carbon monoxide might compete with ivacaftor for binding to this site. Alternatively, the interaction of ivacaftor with this site might modulate allosterically the interaction of CO with CFTR. In support of this latter idea, ivacaftor promotes occupancy of the open channel configuration, providing an explanation for its rescue of CF mutants located throughout the structure of CFTR (26, 27, 73).

Previous work has identified two general mechanisms of CFTR inhibition: open-channel blockade and allosteric inhibition (23, 33, 35). Open-channel blockers, such as glibenclamide and GlyH-101, occlude the vestibules of the CFTR pore, leading to voltage-dependent block (44, 62). By contrast, allosteric inhibitors, such as CFTR_{inh}-172 and (*R*)-

BPO-27 interfere with channel gating (30, 31). Like genistein and thyroid hormones (6, 32), CORM-2 had complex effects on the single-channel behavior of CFTR, exhibiting characteristics of both allosteric inhibition and open-channel blockade. It principally inhibited CFTR by slowing channel gating, influencing gating kinetics in a similar manner to elevated concentrations of genistein (32). However, unlike genistein and (*R*)-BPO-27 (30, 32), elevating the ATP concentration failed to relieve CFTR inhibition. This result suggests that like CFTR_{inh}-172 (31), CORM-2 interferes with channel gating at a different location within the CFTR gating pathway, the wave of conformational changes initiated by ATP binding at the NBD1:NBD2 interface, which lead to opening of the channel pore (13, 64). As indicated above, CORM-2 might exert its effects on channel gating at the ivacaftor-binding site (37, 88). This site is distinct from the CFTR_{inh}-172-binding site, which involves R347, a non-pore lining residue within M6 (5, 23).

The CORM-2-induced reduction in *i* has characteristics of “very fast” open-channel block of CFTR resembling the kinetics of CFTR inhibition by niflumic acid and furosemide (28, 59). However, like elevated concentrations of genistein (32), CORM-2 inhibition of CFTR was voltage-independent. One possibility is that CORM-2 exerts its effects on CFTR by interacting with multiple binding sites. Consistent with this idea, we interpreted the inhibitory effects of genistein on channel gating and current flow to involve its interaction with two sites on CFTR: the NBDs and the intracellular vestibule of the channel pore (32). However, the location of the ivacaftor-binding site (37, 88) raises the interesting possibility that CORM-2 might impede channel gating and anion movement through CFTR by interaction with a single site located in a pivotal position within the MSDs.

The mechanism of CFTR inhibition by CORM-2 appears distinct from its actions on other ion channels. Previous work demonstrates that CORM-2 regulates ion channels by direct and indirect mechanisms [for review, see (85)]. Direct mechanisms include CO binding to histidine residues [e.g. (80)] and a high-affinity, channel-associated heme moiety [e.g. (24)]. Indirect mechanisms include CO-dependent generation of cGMP [e.g. (55)], similar to the action of NO (1) and modulation of the cellular redox state [e.g. (60)]. The former mechanism would be expected to activate CFTR in intestinal epithelial cells (17, 71), but studies using inhibitors of cGMP signaling have proved inconclusive (66, 70). Interestingly, Kapetanaki et al. (29) demonstrated that CO activation of ATP-sensitive K⁺ channels involves heme binding to a CXXHX₁₆H motif on sulphonylurea receptor 2A, an ABC transporter closely related to CFTR. The authors showed that CFTR lacks this heme-binding motif. Similarly, the ivacaftor-binding site (37, 88) lacks amino acid residues previously demonstrated to interact with CO (80), raising the possibility of CO directly regulates CFTR at a different site or by a different mechanism.

This work has several potential caveats. First, we did not test the effects of CO gas on CFTR. Instead, we used the CO-releasing molecules CORM-2 and CORM-3 and found that they had distinct inhibitory effects. As discussed above, although it is well established that the biological activity of CO-releasing molecules is mediated by CO (41, 43), our data do not exclude the possibility that the CO-releasing molecules, themselves, not CO, inhibit CFTR. Second, we did not measure CO generation by CORM-2 and CORM-3. However, we used both CO-releasing molecules at concentrations equivalent to those employed in previous studies of CO's action on epithelial anion transport (48, 66, 70, 89) and demonstrated to liberate CO similar to the original descriptions of these agents (CORM-2: 0.7 mole CO per mole CORM-2; CORM-3: 1 mole CO per mole CORM-3) (11, 42, 89). Although the CO

concentration in epithelial cells is difficult to determine (66), these concentrations are similar to those estimated for endogenous CO production by humans under physiological conditions (~18 $\mu\text{mole CO h}^{-1}$) (12, 57). Third, we examined the direct effects of CO-releasing molecules on the single-channel activity of CFTR. We did not study their action on a large population of CFTR Cl^- channels. In previous work, we have demonstrated that the single-channel behavior of CFTR provides molecular explanations for quantitative changes in CFTR-mediated transepithelial Cl^- currents caused by CF variants [e.g. (61)] and small molecules [e.g. (59)]. Although a further limitation of this work is the use of C127 cells heterologously expressing CFTR, single-channel studies of epithelial cells endogenously expressing human CFTR are highly challenging preventing their routine use.

There are several important implications of the present study. First, the present data and previous results (66, 70, 89) suggest that CO might be a physiological regulator of CFTR function. The CO producing enzymes heme oxygenase-1 (HO-1) and -2 are expressed in epithelial tissues (66, 89). Of note, HO-1 is a modifier gene for *Pseudomonas aeruginosa* infection in CF (46), while in macrophages ezrin appears to assemble HO-1 and CFTR into a macromolecular signaling complex (15, 20). However, the action of CO appears to be tissue-specific, stimulating Cl^- secretion in the intestine (66, 70) and epididymis (48), Na^+ absorption in the kidney (81), but inhibiting ion transport in the lung (2, 89). These distinct effects of CO on epithelial ion transport might be explained by differences in the redox status of cells (85).

Second, the present results and other data (2) suggest that CO should be used with caution to treat lung inflammation. Based on its wide ranging beneficial effects, including dampening inflammation, reducing oxidative stress and strengthening host defense

mechanisms [for review, see (14, 43)], CO has been investigated as a therapy for lung inflammation in acute respiratory distress syndrome (16) and chronic obstructive pulmonary disease (COPD) (3). Like COPD (51), CF lung disease is characterized by persistent bacterial infection and exaggerated inflammatory responses (54). Although CO has been proposed as an adjuvant therapy for CF lung disease (14), our observation that CORM-2 potently inhibits CFTR cautions that great care should be exercised with its use because of the adverse effects on host defense mechanisms of CFTR inhibition (67).

Third, the present results potentially expand the clinical utility of ivacaftor. Currently, ivacaftor is used to treat CF patients with some gating mutations [e.g. G551D; (53)] and in combination with lumacaftor or tezacaftor and elexacaftor, CF patients with the F508del mutation (21, 40, 68, 75). Raju et al. (52) demonstrated that ivacaftor abrogates acquired CFTR inhibition by cigarette smoke, suggesting that the drug might be used to treat COPD and other smoking-related diseases (63). Building on this study, the present results suggest that ivacaftor might be used to alleviate CO poisoning caused environmental pollution.

In conclusion, this study demonstrates that the CO-releasing molecules CORM-2 and CORM-3 inhibit the CFTR Cl^- channel. CORM-2 and CORM-3 predominantly act as allosteric inhibitors, slowing the rate of channel opening. But, additionally, CORM-2 impeded current flow through open channels with very fast kinetics. Of note, the clinically-approved CFTR potentiator ivacaftor abolished CFTR inhibition by CORM-2. Building on other studies (48, 66, 70, 89), the present work suggests that CO might be a physiological regulator of CFTR-mediated epithelial ion transport. The current data and those of Raju et al. (52) also argue that ivacaftor has wider clinical utility than the treatment of CF.

AUTHOR CONTRIBUTIONS

Conception and design of the experiments: W.J.W., N.C. and D.N.S.; performed the research: M.R., W.J., D.R.S.N., B.S.J.H. and J.L.; analysis and interpretation of data: M.R., W.J., D.R.S.N., B.S.J.H., J.L., W.J.W., N.C. and D.N.S.; drafting the article or revising it critically for important intellectual content: M.R., W.J., W.J.W., N.C. and D.N.S.. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this manuscript.

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AUTHOR’S PRESENT ADDRESSES

W. J. Wilkinson: Beechen Cliff School, Kipling Avenue, Bath BA2 1HL, UK

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FIGURE LEGENDS

Figure 1: CORM-2 inhibits the single-channel activity of CFTR (A) Representative single-channel recordings of wild-type human CFTR in an excised inside-out membrane patch from a C127 cell heterologously expressing CFTR in the absence and presence of the indicated concentrations of CORM-2 added acutely to the intracellular solution. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution. Dotted lines indicate the closed channel state and downward deflections correspond to channel openings. Unless otherwise indicated in this and other figures, membrane voltage was clamped at -50 mV, a large Cl^- concentration gradient was imposed across the membrane patch ($[\text{Cl}^-]_{\text{int}}$, 147 mM; $[\text{Cl}^-]_{\text{ext}}$, 10 mM) and temperature was 37°C . (B) Single-channel current amplitude histograms for the 10-s recordings shown in A. For CORM-2 (1 μM), a small leak current shifted the current amplitude histogram by ~ -0.6 pA relative to that of the control. (C and D) Concentration-response relationships for CORM-2 inhibition of single-channel current amplitude (i) and open probability (P_o) determined from prolonged recordings (≥ 5 minutes) acquired using the conditions described in A. Data are means $\pm$ SEM ($n = 6 - 17$). The continuous lines are the fit of sigmoidal 3 parameter functions to mean data.

Figure 2: Ruthenium chloride is without effect on the single-channel behavior of CFTR

(A) Representative single-channel recordings of wild-type human CFTR in an excised inside-out membrane patch from a C127 cell heterologously expressing CFTR in the absence and presence of RuCl_3 (100 μM) added acutely to the intracellular solution. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution. Dotted lines indicate the closed channel state and downward deflections correspond to channel openings. (B and C) Summary single-channel current amplitude (i) and open probability (P_o) data determined

from prolonged recordings (≥ 5 minutes) show the effects of RuCl_3 (100 μM) on wild-type human CFTR. Symbols represent individual values and columns means $\pm$ SEM ($n = 5$).

Figure 3: CORM-3 inhibits the single-channel activity of CFTR (A) Representative single-channel recordings of wild-type human CFTR in an excised inside-out membrane patch from a C127 cell heterologously expressing CFTR in the absence and presence of the indicated concentrations of CORM-3 and CFTR_{inh}-172 added acutely to the intracellular solution. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution. Dotted lines indicate the closed channel state and downward deflections correspond to channel openings. (B) Single-channel current amplitude histograms for the 10-s recordings shown in A. With the exception of CORM-3 (300 μM) where the leak current was ~ -4.8 pA, in the presence of CORM-3 a small leak current shifted the current amplitude histogram by ~ -0.6 pA relative to that of the control. (C and D) Summary single-channel current amplitude (i) and open probability (P_o) data determined from prolonged recordings (≥ 5 minutes) show the effects of CORM-3 (30 – 300 μM) and CFTR_{inh}-172 (I172; 10 μM) on wild-type human CFTR. Symbols represent individual values and columns means $\pm$ SEM ($n = 5 - 6$); **, $P < 0.01$; ***, $P < 0.001$ vs control.

Figure 4: Dwell time histograms of a single CFTR Cl^- channel inhibited by CORM-2 (A and B) Representative dwell-time histograms for a single wild-type human CFTR Cl^- channel recorded in the absence (A) and presence (B) of CORM-2 (30 μM) in the intracellular solution; ATP (0.3 mM) and PKA (75 nM) were present throughout the recordings. The continuous lines are the fit of one-, two- or four-component exponential functions to the data and the dashed lines show the individual components of these functions. The vertical dashed

lines indicate mean values of the different time constants (for details, see Table 1).
Logarithmic x -axes with 10 bins decade⁻¹ were used for dwell-time histograms.

Figure 5: CFTR inhibition by CORM-2 is voltage-independent (A) Representative recordings of two wild-type human CFTR Cl⁻ channels in an excised inside-out membrane patch from a C127 cell heterologously expressing CFTR. The recordings were acquired at ± 50 mV in the absence and presence of CORM-2 (30 μ M). ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution and the membrane patch was bathed in symmetrical 147 mM Cl⁻ solutions. Dotted lines indicate the closed channel state and downward deflections at -50 mV and upward deflections at $+50$ mV correspond to channel openings, identified by the labels O1 and O2. (B and C) Summary single-channel current amplitude (i) and open probability (P_o) data show the effects of CORM-2 (30 μ M) on wild-type human CFTR at ± 50 mV. Symbols represent individual values and columns means $\pm$ SEM ($n = 6$); **, $P < 0.01$ vs. control.

Figure 6: The ATP-dependence of CFTR inhibition by CORM-2 (A and B) Representative recordings of wild-type human CFTR Cl⁻ channels in excised inside-out membrane patches from C127 cells heterologously expressing CFTR in the absence and presence of CORM-2 (30 μ M) when the ATP concentration was either 0.3 mM (A) or 3 mM (B). ATP (0.3 or 3 mM) and PKA (75 nM) were continuously present in the intracellular solution. Dotted lines indicate the closed channel state and downward deflections correspond to channel openings. (C and D) Summary single-channel current amplitude (i) and open probability (P_o) data determined from prolonged recordings (≥ 5 minutes) show the effects of CORM-2 (30 μ M) on wild-type human CFTR when the intracellular solution contained either

0.3 or 3 mM ATP. Symbols represent individual values and columns means $\pm$ SEM ($n = 6 - 8$); *, $P < 0.05$ vs. control at same [ATP].

Figure 7: Ivacaftor impedes CFTR inhibition by CORM-2 (A) Representative single-channel recordings of wild-type human CFTR in an excised inside-out membrane patch from a C127 cell heterologously expressing CFTR under the indicated experimental conditions. Small molecules were acutely added to the intracellular solution in the continuous presence of ATP (0.3 mM) and PKA (75 nM). Dotted lines indicate the closed channel state and downward deflections correspond to channel openings. (B – E) Summary single-channel current amplitude (i), open probability (P_o), mean burst duration (MBD) and interburst interval (IBI) data determined from prolonged recordings (≥ 5 minutes) show the effects CORM-2 (30 μ M) and CFTR_{inh}-172 (10 μ M) on wild-type human CFTR potentiated by ivacaftor (100 nM); compounds were added sequentially and cumulatively. Symbols represent individual values and columns means $\pm$ SEM ($n = 4 - 6$); **, $P < 0.01$; ***, $P < 0.001$ vs. control.

Figure 8: Ivacaftor relieves CFTR inhibition by CORM-2 (A and B) Summary single-channel current amplitude (i) and open probability (P_o) data determined from prolonged recordings (≥ 5 minutes) show the effects of ivacaftor (100 nM) and CFTR_{inh}-172 (10 μ M) on wild-type human CFTR inhibited by CORM-2 (30 μ M); compounds were added sequentially and cumulatively. Symbols represent individual values and columns means $\pm$ SEM ($n = 5$); **, $P < 0.01$; ***, $P < 0.001$ vs. control.

993 **TABLES**

994 **Table 1: Effects of CORM-2 on the open- and closed-time constants of wild-type human**

995 **CFTR**

	[CORM-2] (μM)	
[Drug]	0	30
<i>n</i>	3	3
τ_{O1} (ms)	-----	11.4 ± 5.2
τ_{O2} (ms)	35.7 ± 8.8	42.6 ± 24.6
τ_{C1} (ms)	-----	0.75 ± 0.49
τ_{C2} (ms)	2.22 ± 0.04	3.51 ± 0.71
τ_{C3} (ms)	196 ± 44	115 ± 32
τ_{C4} (ms)	-----	$2,225 \pm 1,255$
Area under curve τ_{O1}	-----	0.67 ± 0.14
Area under curve τ_{O2}	1	0.33 ± 0.14
Area under curve τ_{C1}	-----	0.51 ± 0.23
Area under curve τ_{C2}	0.75 ± 0.05	0.37 ± 0.19
Area under curve τ_{C3}	0.25 ± 0.05	0.11 ± 0.04
Area under curve τ_{C4}	-----	0.01 ± 0.01
Events per minute	$1,136 \pm 204$	$1,071 \pm 267$
Total time (s)	1,442	845

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Table 1: Effects of CORM-2 on the open- and closed-time constants of wild-type human CFTR Open- and closed-time constants were measured at the indicated concentrations of CORM-2 by fitting one-, two- or four-component exponential functions to open- and closed-time histograms. Areas under curve indicate the proportion of the total dwell time distribution that correspond to the different time constants. Events per minute represents the number of transitions between the open and closed states within one minute. The total time analyzed in the absence and presence of CORM-2 (30 μ M) is shown and in each membrane patch ~5,000 events were analyzed per intervention. Values are means $\pm$ SEM of n observations. Measurements were made in the presence of the catalytic subunit of PKA (75 nM) and ATP (0.3 mM) in the intracellular solution. Voltage was -50 mV, there was a large Cl^- concentration gradient across the membrane patch ($[\text{Cl}^-]_{\text{internal}} = 147$ mM; $[\text{Cl}^-]_{\text{external}} = 10$ mM) and temperature was 37°C .

Figure 1

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