A role for the cystic fibrosis transmembrane conductance regulator in the nitric oxide-dependent release of Cl⁻ from acidic organelles in amacrine cells

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A role for the cystic fibrosis transmembrane conductance regulator in the nitric oxide-dependent release of Cl\(^{-}\) from acidic organelles in amacrine cells

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Krishnan V, Maddox JW, Rodriguez T, Gleason E. A role for the cystic fibrosis transmembrane conductance regulator in the nitric oxide-dependent release of Cl\(^{-}\) from acidic organelles in amacrine cells. J Neurophysiol 118: 2842–2852, 2017. First published August 23, 2017; doi:10.1152/jn.00511.2017.—γ-Amino butyric acid (GABA) and glycine typically mediate synaptic inhibition because their ligand-gated ion channels support the influx of Cl\(^{-}\). However, the electrochemical gradient for Cl\(^{-}\) across the postsynaptic plasma membrane determines the voltage response of the postsynaptic cell. Typically, low cytosolic Cl\(^{-}\) levels support inhibition, whereas higher levels of cytosolic Cl\(^{-}\) can suppress inhibition or promote depolarization. We previously reported that nitric oxide (NO) releases Cl\(^{-}\) from acidic organelles and transiently elevates cytosolic Cl\(^{-}\), making the response to GABA and glycine excitatory. In this study, we test the hypothesis that the cystic fibrosis transmembrane conductance regulator (CFTR) is involved in the NO-dependent efflux of organellar Cl\(^{-}\). We first establish the mRNA and protein expression of CFTR in our model system, cultured chick retinal amacrine cells. Using whole cell voltage-clamp recordings of currents through GABA-gated Cl\(^{-}\) channels, we examine the effects of pharmacological inhibition of CFTR on the NO-dependent release of internal Cl\(^{-}\). To interfere with the expression of CFTR, we used clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing. We find that both pharmacological inhibition and CRISPR/Cas9-mediated knockdown of CFTR block the ability of NO to release Cl\(^{-}\) from internal stores. These results demonstrate that CFTR is required for the NO-dependent efflux of Cl\(^{-}\) from acidic organelles.

NEW & NOTEWORTHY Although CFTR function has been studied extensively in the context of epithelia, relatively little is known about its function in neurons. We show that CFTR is involved in an NO-dependent release of Cl\(^{-}\) from acidic organelles. This internal function of CFTR is particularly relevant to neuronal physiology because postsynaptic cytosolic Cl\(^{-}\) levels determine the outcome of GABA- and glycinergic synaptic signaling. Thus the CFTR may play a role in regulating synaptic transmission.

CFTR; cytosolic chloride; amacrine cells; nitric oxide

THE REGULATION OF CYTOSOLIC and organellar Cl\(^{-}\) is critical to normal cellular physiology (reviewed in Faundez and Hartzell 2004; Stauber and Jentsch 2013). In neurons, cystosolic Cl\(^{-}\) has the additional role of determining the sign of GABA- and glycinergic synapses. When postsynaptic cytosolic Cl\(^{-}\) levels are low and the equilibrium potential for Cl\(^{-}\) is quite negative, GABA and glycine will cause inhibition. If postsynaptic cytosolic Cl\(^{-}\) levels are elevated, however, inhibition is either reduced or is converted to excitation (Kaila et al. 2014). Amacrine cells are retinal interneurons that receive depolarizing input from glutamatergic bipolar cells and make primarily inhibitory GABA- and glycinergic synapses onto retinal ganglion cells that then send the retinal signal to higher visual centers. The most common synaptic interactions for amacrine cells, however, are with other amacrine cells (Marc and Liu 2000). Additionally, amacrine cell dendrites can have adjacent pre- and postsynaptic sites, making regulation of local Cl\(^{-}\) levels especially important.

Our laboratory demonstrated that nitric oxide (NO) can elicit the release of Cl\(^{-}\) from acidic organelles (Hoffpaur et al. 2006; Krishnan and Gleason 2015) and that this release is itself dependent on transient cystosolic acidification (McMains and Gleason 2011). However, the transporter(s) mediating this NO-dependent internal Cl\(^{-}\) release has remained elusive. Many of the pharmacological modulators of Cl\(^{-}\) channels and transporters lack specificity or are unavailable, making the mediators of this Cl\(^{-}\) efflux pathway challenging to identify. The cystic fibrosis transmembrane conductance regulator (CFTR) is a good candidate for involvement in this mechanism, because CFTR currents can be potentiated by cystosolic acidification (Chen et al. 2009).

The CFTR is a member of the ATP binding cassette transporter family that functions as an ATP- and phosphorylation-dependent anion channel that supports the flux of Cl\(^{-}\) (reviewed in Hwang and Sheppard 2009). This channel has been extensively studied in the context of epithelia because of the deleterious effects of its mutated forms in cystic fibrosis (Riordan et al. 1989). The most common mutation, ΔF508, leads to abnormal folding of the protein and reduced trafficking to the epithelial apical plasma membrane (Wang and Li 2014). CFTR expression is not, however, confined to epithelia. For example, it is expressed in cardiovascular system in cell types such as cardiac myocytes (Gadsby et al. 1995), endothelial cells (Tousson et al. 1998), and immune cells (Di et al. 2006; Painter et al. 2006). Other examples of CFTR-expressing cell types include smooth muscle (Vandebrouck et al. 2006), skeletal muscle (Tu et al. 2010), osteoblasts (Shead et al. 2007), and neurons (Guo et al. 2009a, 2009b; Mulberg et al. 1995).

The limited number of functional studies in neurons demonstrate a role for CFTR in regulating cytosolic Cl\(^{-}\) (Morales et al. 2011; Ostroumov et al. 2011), nonvesicular ATP release (Kanno and Nishizaki 2011), glucose sensing by hypothalamic

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neurons (Chalmers et al. 2014; Murphy et al. 2009), exocytosis (Weyler et al. 1999), and peripheral nerve myelination (Reznikov et al. 2013). In addition to its well-established expression at the plasma membrane, CFTR can be active in multiple acidic organelles, including the trans-Golgi (Barasch et al. 1991), endosomes (Biweri and Verkman 1994), and lysosomes (Liu et al. 2012).

To explore the involvement of CFTR in the NO-dependent release of Cl⁻ from acidic organelles, we first investigated the expression of CFTR in retinal amacrine cells by looking for the presence of both mRNA and protein. We then examined the effects of selective pharmacological inhibition of CFTR function. Finally, we determined the consequences of inhibiting the expression of CFTR with a CRISPR/Cas9 gene editing strategy. Our results demonstrate that CFTR is expressed by amacrine cells and that it is required for the NO-dependent release of Cl⁻ from acidic organelles.

MATERIALS AND METHODS

All methods involving animals have been approved by the Louisiana State University Institutional Animal Care and Use Committee.

Cell culture. Retinal cultures were derived from chick embryos by using the methods described in Krishnan and Gleason (2015). For experiments involving CFTR inhibitors (see Figs. 2 and 3), retinas were dissociated from 8-day chick embryos. For the experiments involving CRISPR/Cas9 gene editing (see Figs. 4 and 5), cells dissociated from 11-day chick embryos were used because they appeared healthier after transfection. In either case, recordings were made from cultures on embryonic equivalent days 15–18. No physiological differences between cells cultured on either time frame were detected in these experiments. Cells were plated on poly-l-ornithine–treated dishes at a density of 2.5 × 10⁵ cells per 35-mm dish. Retinal cells were initially plated in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 5% fetal calf serum (Sigma, St. Louis, MO) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Life Technologies). One day after plating, DMEM was replaced with Neurobasal medium (Life Technologies) supplemented with 1% B-27 nutrient medium and penicillin-streptomycin-glutamine (Life Technologies). Cells were fed every other day for 2 weeks and then properly disposed of. Amacrine cells were identified on the basis of their morphology as previously described (Gleason et al. 1993).

RT-PCR. Pools of five amacrine cells from culture dishes rinsed in Hanks’ solution were collected by aspiration into siliconized (Sigma) glass micropipettes. The pipette tips were broken in PCR Hanks’ solution were collected by aspiration into siliconized (Sigma) glass micropipettes. The pipette tips were broken in PCR 5% normal goat serum in dilution solution (1% BSA-0.5% saponin in PBS) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue, and 62.5 mM Tris. Protein samples (20 μg) were prepared from chicken brains as previously reported (McMains and Gleason 2011). Before electrophoresis, protein samples were prepared in Laemml buffer containing 2% SDS, 5% 2-metacaptoethanol, 10% glycerol, 0.005% bromophenol blue, and 62.5 mM Tris. Protein samples (20 μg) in Laemml buffer were incubated at room temperature for 1 h. Proteins were separated on an 8% bis-acrylamide–SDS gel with 10 μl of PageRuler prestained protein ladder (ThermoFisher Scientific). The membranes were blocked with 5% normal goat serum and 1% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 2 h. Block solution was replaced with the diluted primary anti-CFTR antibody (1:1,000) in TBS-T with 1% BSA and incubated for 2 h. The AbExcel goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase (catalog no. ab97051; Abcam) was diluted (1: 50,000) in TBS-T with 1% BSA. The membrane was incubated with the secondary antibody for 1 h and then washed in TBS-T. Western blot analysis. Protein samples were prepared from chicken brains as previously reported (McMains and Gleason 2011). Before electrophoresis, protein samples were prepared in Laemml buffer containing 2% SDS, 5% 2-metacaptoethanol, 10% glycerol, 0.005% bromophenol blue, and 62.5 mM Tris. Protein samples (20 μg) in Laemml buffer were incubated at room temperature for 1 h. Proteins were separated on an 8% bis-acrylamide–SDS gel with 10 μl of PageRuler prestained protein ladder (ThermoFisher Scientific). Separated proteins were transferred onto nitrocellulose membrane at 4°C overnight. The following steps were carried out at room temperature. The membranes were blocked with 5% normal goat serum and 1% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 2 h. Block solution was replaced with the diluted primary anti-CFTR antibody (1:1,000) in TBS-T with 1% BSA and incubated for 2 h. The AbExcel goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase (catalog no. ab97051; Abcam) was diluted (1: 50,000) in TBS-T with 1% BSA. The membrane was incubated with the secondary antibody for 1 h and then washed in TBS-T. Western blot images were captured using Image Lab software (Bio-Rad).
glass bridge. Patch pipettes with tip resistance values of 8–11 MΩ were pulled from thick-walled borosilicate glass (Sutter Instruments, Novato, CA) using a P-97 micropipette puller (Sutter Instruments). Electrophysiology experiments were performed on amacrine cells in the whole cell voltage-clamp mode using Axopatch-1D patch clamp (Molecular Devices, Sunnyvale, CA) and Clamper 9.2 software (Molecular Devices). Whole cell data were filtered by the voltage-clamp amplifier at 2 kHz and digitized at 5 kHz with either a Digidata 1322A or Digidata 1440A (Molecular Devices). Recordings were rejected if the membrane resistance fell below 500 MΩ. Series resistance (typical range 10–25 MΩ) values were monitored throughout the experiments and were typically stable. If large changes (>20%) did occur, the data were discarded. For voltage-ramp experiments, cells were held at ~70 mV and a voltage ramp was delivered (~90 to +50 mV over 200 ms) first in the absence of GABA and then repeated in the presence of GABA. Before leak subtraction, corrections were made for errors due to the liquid junction potential (estimated using the pClamp calculator, −13 mV) and the series resistance (as estimated by Clamper). Data were leak-subtracted by subtracting the current in response to the first voltage ramp from the current in response to the second voltage ramp and in the presence of GABA. The electrophysiological evaluation of transfected and control amacrine cells was done on amacrine cells derived from four separate transfections. Experiments were carried out at room temperature.

Solutions. Unless otherwise specified, reagents were obtained from Sigma-Aldrich. For electrophysiological recordings conducted under Cl−-free conditions (see Fig. 2), the external solution contained (in mM) 145.0 Na-methanesulfonate, 5.6 glucose, and 10.0 HEPES. External solutions were adjusted to pH 7.4 with NaOH. The internal zero-Cl− solution contained (in mM) 145.0 Cs-methanesulfonate and 10.0 HEPES. The outflow was positioned near the KCl-containing bridge to minimize any effect of Cl− leaking from the bridge into the bath. Voltage-ramp experiments were done in tetraethylammonium (TEA-Cl) external solution containing (in mM) 116.7 NaCl, 5.3 KCl, 20.0 TEA-Cl, 3.0 CaCl2, 0.41 MgCl2, 10.0 HEPES, and 5.6 glucose. The TEA-Cl external solution was supplemented with 300 mM TTX-Sigma-Aldrich. For electrophysiological recordings conducted under NO-saturated conditions (see Fig. 2), the external solution contained (in mM) 145.0 Cs-methanesulfonate and 10.0 HEPES. The outflow was positioned near the KCl-containing bridge to minimize any effect of NO leaking from the bridge into the bath.

Voltage-ramp experiments were done with perfusion via a Perfusion Pencil (AutoMate, Berkeley, CA). Experiments involving voltage ramps during exposure to GABA or other ligands were done with perfusion through a ValveLink pinch valve pressurized perfusion system (Automate Scientific, Berkeley, CA). For GABA “pulse” experiments (see Fig. 2), solution changes were achieved with a computer-controlled automated SF-77B perfusion fast stepper (Warner Instruments, Hamden, CT). Experiments involving voltage ramps during exposure to GABA were done with perfusion via a Perfusion Pencil (AutoMate, Berkeley, CA) positioned near the recorded cell. As a control, cells were routinely exposed to NO-free, low-pH solution (McMains and Gleason 2011), and none of the results reported in this article were reproduced using this treatment. NO was prepared by bubbling Fisher ultra-pure water for 15 min with argon, followed by bubbling with pure NO gas for 15 min. NO (30–50 μM) was injected into the perfusion line and has been previously estimated to reach the recorded cell in ~3 s and to remain for ~3–5 s (Hoffpauir et al. 2006).

CRISPR/Cas9. To genetically knockdown CFTR, we employed the CRISPR/Cas9 system, developed by Ran et al. (2013), to introduce insertions/deletions (indels) into the CFTR gene and disrupt CFTR protein expression. The plasmid pSpCas9(BB)-2A-GFP was a gift from Feng Zhang (Addgene plasmid no. 48138). The single guide (sg)RNA was designed to target the 7th exon of the CFTR gene using the web tool CRISPR (https://crispr.mit.edu). The corresponding amino acid sequence includes intracellular loop 2, which is reported to interact with one of the nucleotide binding domains (Mornon et al. 2008). Mutational analysis reveals roles in channel activity with a 19-amino acid deletion producing alterations in conductance states (Xie et al. 1995). Missense mutations introduced into intracellular loop 4, which also interacts with nucleotide binding domains, produce changes in CFTR channel gating (Cotten et al. 1996). Oligos were synthesized by Integrated DNA Technologies (Coralville, IA) and inserted into pSpCas9(BB)-2A-GFP according to Ran et al. (2013) to generate pCRISPR-CFTR. The primers used to generate the sgRNA were CFTR E7 forward, caccgCTATTGCTGGGAAGATGCAA and CFTR E7 reverse, aaacctGATCCATTTCCACGAAAT. Transfections were performed using the Amaxa Nucleofector 2b from Lonza (Basel, Switzerland). Aliquots of 8 × 104 retina cells in suspension were transfected according to the manufacturer’s protocol. Transfection efficiency was ~10–20%. Cells transfected with (BB)-2A-GFP (no sgRNA) served as a negative control along with nontransfected cells.

To detect indels (insertions/deletions) in the 7th exon of CFTR by nucleomonomorphing end joining induced by Cas9-generated double-stranded DNA cleavage, genomic DNA (gDNA) from single transfected dishes or single transfected amacrine cells was isolated using the Arcturus PicoPure kit (ThermoFisher Scientific). The 7th exon of CFTR was PCR amplified using Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and primers that were designed to anneal in regions flanking the 7th exon (forward, AGTTGCTGTGTTAGTGCTTG; reverse, GCCGTGTACTTATGAGAG). Correct PCR amplification was verified by sequencing (Eurofins Genomics, Louisvile, KY). THRecA (New England Biolabs), along with its cofactor ATP, was added to the amplification mix to improve specificity and yield (Shigemori et al. 2005). PCR products (394 bp) from pCRISPR-CFTR transfections and pSpCas9 transfections were used in the T7 endonuclease I (T7EI) mutation detection assay (New England Biolabs), which recognizes and cleaves mismatched double-stranded DNA. PCR products from each gDNA amplification were denatured and slowly reannealed to allow heteroduplex formation. Each heteroduplex DNA sample was digested with T7E1 (10 units) for 30 min at 37°C. T7E1-digested DNA (50 ng) was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using the ChemiDoc XRS + software (Bio-Rad). Images were captured and analyzed using Image Lab software (Bio-Rad).

To confirm that the level of CFTR protein was diminished by the CRISPR/Cas9 edit, CFTR antibody labeling was evaluated for transfected and control amacrine cells. Green fluorescent protein (GFP) fluorescence in cells transfected with pCRISPR-CFTR did not withstand fixation, making identification of transfected cells unreliable. To overcome this, the GFP gene in pSpCas9(2A)-GFP was replaced with tdTomato gene from the plasmid tdTomato-N1 to create pSpCas9tdT. The tdTomato-N1 was a gift from Michael Davidson and Roger Tsien (unpublished; Addgene plasmid no. 54642). As described above, the sgRNA targeting Cas9 to the 7th exon of CFTR was then inserted into pSpCas9tdT to create pCRISPR-CFTRtdT. Cells transfected with either pSpCas9tdT or pCRISPR-CFTRtdT were fixed and immunolabeled for CFTR as described above.

Data analysis. CFTR labeling of transfected and control amacrine cell bodies was analyzed using the image processing software (Intelligent Imaging Innovations). A lower threshold mask corresponding to the CFTR channel was created based on nontransfected and secondary-only cell images. The CFTR mask was then applied to images of cells transfected with pSpCas9tdT and pCRISPR-CFTRtdT (see Fig. 4). The relatively thick cell bodies were removed from the CFTR mask in all images. The CFTR mask was also removed from any areas of the images that did not have any tdTomato fluorescence (arising from nontransfected cells in the dish). Only processes with tdTomato ex-
expression were used for analysis. SlideBook software automatically calculated CFTR mask descriptive statistics for CFTR object number (>2 pixels), CFTR object area, CFTR sum intensity, and tdTomato sum intensity. Welch’s t-tests were performed on all mask data using Prism 7 (GraphPad Software, La Jolla, CA). The analysis was done separately for cell bodies. Other data were analyzed as follows. For multiple comparisons, the one-way ANOVA with Tukey’s post hoc multiple comparisons test (see Figs. 3 and 5) was used. Pairwise comparisons were made with the paired t-test (see Fig. 2). Data are means ± SE. The P value for significance was <0.05.

RESULTS

To determine whether CFTR is expressed by amacrine cells, mRNA from pools of five amacrine cells was amplified by RT-PCR using CFTR gene-specific primers. A single PCR product was produced at the correct size (121 bp; Fig. 1A), and sequencing confirmed that it was from CFTR. To determine whether the CFTR protein is expressed, a polyclonal antibody raised against human CFTR was identified that recognizes a single band of the appropriate molecular mass (168 kDa) in chicken brain homogenate (Fig. 1B). In culture, the antibody labeled amacrine cells both at the cell body and in neuronal processes (Fig. 1C). Cone photoreceptor cells were also present in our cultures, and although they lack outer segments, they are easily identifiable by their characteristic morphology and the possession of an oil droplet (Fig. 1D, arrow) (Adler et al. 1984; Wilson and Gleason 1991). These cells had a distinctive labeling pattern where CFTR immunoreactivity was especially concentrated in the ellipsoid region of the cell, a domain rich with mitochondria (Fig. 1D, asterisk). Presumably, this pattern reflects a strong localization of CFTR to mitochondrial membranes.

In sections of adult chicken retina, strong CFTR labeling was also seen in photoreceptor ellipsoids (Fig. 1E) as well as in the outer plexiform layer, where synapses between photoreceptors and horizontal and bipolar cells are found. Some CFTR labeling can be seen throughout the inner nuclear layer where horizontal, bipolar, and amacrine cell bodies reside. In this layer, the most distinctive labeling is in the inner one-third to one-half, where amacrine cell bodies predominate. Labeling was found throughout the inner plexiform layer, where amacrine as well as bipolar cells make their synapses. Additionally, there was a band of more intense labeling near the outer margin of the inner plexiform layer (Fig. 1E, arrowheads). Cells in the ganglion cell layer as well as the ganglion cell axons were also labeled. Together, these results indicate that the CFTR protein is expressed by amacrine cells both in culture and in the intact retina. Furthermore, CFTR is broadly expressed in the retina and thus has the potential to influence retinal function at multiple sites.

To investigate the role of the CFTR in the NO-dependent release of Cl− from acidic organelles, experiments were done in the absence of extracellular and intracellular (pipette) Cl− to isolate the intracellular store as the only source of Cl−. Wash-out of cytosolic Cl− was verified by the absence of a response to pulses of GABA (20 μM; Fig. 2A, top left) with the cell held at −70 mV. After NO, small GABA-gated inward currents are transiently observed, which represent Cl− released from the internal store and then exiting the cell via the open GABA\(_A\) receptors (Hoffpaur and Gleason 2002). NO-dependent GABA-gated currents disappear during the next recording of five GABA pulses. The effects of two cell-permeable CFTR inhibitors were tested: glibenclamide (20 μM) and CFTR\(_{inh}−\)
Inhibitors of CFTR block the release of Cl⁻ from acidic organelles. A: representative traces from an amacrine cell voltage-clamped at −70 mV with pulses of GABA (20 μM) applied as indicated by the horizontal bars. Recordings were made in Cl⁻-free internal and external solutions. The absence of GABA-gated currents indicates that cytosolic Cl⁻ has washed out. Under control conditions, NO elicits a small, GABA-dependent inward current, which represents newly available cytosolic Cl⁻ exiting through open GABA receptors. In the same cell but in the presence of glibenclamide (20 μM), no GABA-gated currents appear after NO. B: mean current amplitudes before and after NO are plotted for control and glibenclamide data (n = 5). *P < 0.05, paired t-test. C: the same experiment as in A, but data are shown from a different amacrine cell and the effectiveness of CFTRinh-172 (10 μM) was tested. D: mean current amplitudes are plotted for the GABA-gated currents in the presence and absence of CFTRinh-172 (n = 7). ***P < 0.001, paired t-test. Individual data points are plotted with the dotted line indicating the mean and the bars indicating SE.

Glibenclamide functions as an internal pore blocker and is also known to inhibit ATP-dependent K⁺ channels (Sheppard and Robinson 1997; Sheppard and Welsh 1992). CFTRinh-172 affects CFTR channel gating (Kopeikin et al. 2010) and can affect mitochondrial function, although not in the time frame of exposure in these experiments (Kelly et al. 2010). For both inhibitors, cells that had demonstrated NO-dependent release under control conditions were no longer responsive to NO in the presence of the inhibitors (Fig. 2, A and C, bottom right traces). On average, the CFTR inhibitors significantly blocked the ability of NO to generate GABA-gated currents due to release of internal Cl⁻ (control current: 36.2 ± 18.2 pA, n = 5; glibenclamide current: 2.0 ± 1.3 pA, n = 5, P < 0.05, paired t-test; control current: 16.8 ± 2.6 pA, n = 7; CFTRinh-172 current: 1.1 ± 0.6 pA, n = 7, P < 0.001, paired t-test).

To confirm that glibenclamide and CFTRinh-172 were inhibiting the NO-dependent release of Cl⁻, we estimated the Cl⁻ content of the cytosol by measuring the reversal potential of the leak-subtracted GABA-gated current (E_{REV,GABA}) in solutions containing normal Cl⁻ concentrations. Under control conditions, NO caused a shift in E_{REV,GABA} of the current in the positive direction, indicating an increase in cytosolic Cl⁻ (Fig. 3, A and C). In the presence of either glibenclamide or CFTRinh-172, however, the shifts in E_{REV,GABA} were suppressed (control: 26.7 ± 4.5 mV, n = 9; glibenclamide: 1.6 ± 1.2 mV, n = 7; CFTRinh-172: −0.3 ± 0.5 mV, n = 9, P < 0.0001, 1-way ANOVA with Tukey’s multiple comparisons test; Fig. 3F). When the inhibitors were applied first, NO-dependent shifts in E_{REV,GABA} could still be observed after the wash (Fig. 3, Ei–Eiv), indicating that the effects of the inhibitors were at least partially reversible. Another CFTR inhibitor, GlyH101, was also tested, but it inhibited the GABA-gated currents, making measurements of E_{REV,GABA} difficult. This unexpected effect of GlyH101 may be due to its activity as an external Cl⁻ channel pore blocker (Sonawane and Verkman 2003). Nonetheless, the effects of the other inhibitors suggest that CFTR activity is required for the NO-dependent release of Cl⁻ from acidic organelles.

To confirm a role for CFTR, we used a CRISPR/Cas9 gene editing strategy to knock down expression of the protein. To confirm that Cas9 was targeted to the 7th exon of CFTR to induce indels (insertions/deletions), the 7th exon was PCR amplified from either single transfected amacrine cell genomic DNA (Fig. 4Ai) or pooled genomic DNA from cultures (Fig. 4Aii; containing both transfected and nontransfected cells) that were transfected with either pCRISPR-CFTR or pSpCas9. The PCR product (~394 bp) was then analyzed for indels in the CFTR gene using the T7 endonuclease I (T7E1) mutation assay (EnGen Mutation kit; New England Biolabs). T7E1 cleaved heteroduplexed PCR products amplified from cells transfected with pCRISPR-CFTR into appropriate-sized fragments (~228 and ~166 bp) but did not cleave the PCR products amplified from cells transfected with pSpCas9 for samples derived from both transfected individual amacrine cells (Fig. 4Aii) or transfected cultures (Fig. 4Ai). These results demonstrate that pCRISPR-CFTR is effective at targeting Cas9 to the 7th exon of CFTR to induce indels, which lead to the reduction in CFTR expression.

To assess the effectiveness of knocking down CFTR expression by targeting Cas9 to the 7th exon of the CFTR gene with the CFTR-specific sgRNA, amacrine cells transfected without the sgRNA (pSpCas9td) or with the sgRNA (pCRISPR-CFTRtd) were labeled using the same anti-CFTR antibody as
cells that were transfected with pCRISPR-CFTR td had a 69% reduction in the total CFTR-labeled area in processes (pSpCas9 td: 14.58 ± 1.7 μm², n = 10; pCRISPR-CFTR td: 4.5 ± 1.1 μm², n = 10, P = 0.0001, Welch’s t-test; Fig. 4H), a ~34% reduction in CFTR-labeled object size (pSpCas9 td: 71.27 ± 5.74 nm², n = 10; pCRISPR-CFRT td: 47.01 ± 5.24 nm², n = 10, P = 0.006, Welch’s t-test; Fig. 4I), and a 35% reduction in the CFTR object fluorescence intensity within the area of CFTR labeling (CFTR objects) was not significantly different (pSpCas9 td: 4,430 ± 828.4 A.U., n = 10; pCRISPR-CFTR td: 3,365 ± 553.2 A.U., n = 10, P = 0.3, Welch’s t-test; Fig. 4K). Cell bodies were analyzed separately, and CFTR fluorescence intensity was reduced by 18% in the pCRISPR-CFTR td-transfected cells compared with control (pSpCas9 td: 100,994 ± 6,065 A.U., n = 9; pCRISPR-CFTR td: 82,444 ± 5,496 A.U., n = 10, P = 0.04).

To assess the physiological effects of the CFTR gene editing, voltage-clamp recordings were made from amacrine cells transfected with pCRISPR-CFTR, with the construct minus the guide RNA sequence (pSpCas9), GFP-negative cells from a transfection (~GFP), and nontransfected cells. Amacrine cells were recorded from 4–7 days after transfection and plating. $E_{REV-GABA}$ was determined by measuring the leak-subtracted current produced by voltage ramps delivered in the presence of GABA. NO-dependent positive shifts in $E_{REV-GABA}$ were not observed in amacrine cells transfected with pCRISPR-CFTR (mean shift ~3.6 ± 2.7 mV, n = 26; Fig. 5, A and E), but NO-dependent positive shifts in $E_{REV-GABA}$ were observed for all the control cell populations (mean shifts; pSpCas9: 13.5 ± 3.0 mV, n = 22; ~GFP: 14.1 ± 3.5, n = 16; nontransfected: 19.9 ± 4.4 mV, n = 10; Fig. 5, B–D). Mean shift values for cells transfected with the full construct were significantly different from those observed in each of the control cell populations ($P < 0.0001, 1$-way ANOVA, Tukey’s multiple comparisons test; Fig. 5F). In some pCRISPR-CFTR cells, NO produced a negative shift in $E_{REV-GABA}$, suggesting that Cl⁻ is leaving the cytosol. Similar observations were made in the inhibitor experiments (Fig. 3). We currently do not know the mechanism underlying this observation, but it might be a reflection of the interactions between transport mechanisms and an imbalance generated by the reduction in CFTR expression (or function).

**DISCUSSION**

Amacrine cell RT-PCR and immunocytochemistry experiments demonstrate that these cells express CFTR mRNA and protein, respectively. Established inhibitors of CFTR, glibenclamide (Sheppard and Welsh 1992) and CFTR inh-172 (Ma et al. 2002), prevent the release of Cl⁻ from acidic organelles by NO, and a reduction in CFTR expression by using CRISPR/
transfection-related debris was chosen for quantitative analysis to maximize the quality of the data. 0.001, Welch’s repeated on cells from 3 different transfections. Similar qualitative results were obtained from each experiment. However, the transfection with the least Cas9 to disrupt the CFTR gene also prevents NO from releasing internal Cl\(^{-}\). Together these results indicate that CFTR is required for the NO-dependent release of Cl\(^{-}\) from acidic organelles.

Resting our conclusions on pharmacology alone would be less compelling, because although they are well established to inhibit CFTR currents, both glibenclamide and CFTRinh-172 have been shown to inhibit a current that is elicited by parathyroid hormone and is dependent on Cl\(^{-}\). The pharmacology of human CFTR is fairly well worked out, but there is evidence that the pharmacology of this protein differs across species. For example, CFTR\(_{\text{inh}}\)172 has little inhibitory effect on shark CFTR but is effective (in order of declining effectiveness) on human, killi-fish, and pig CFTR (Stahl et al. 2012). Limited information is available on chicken CFTR; however, 300 µM glibenclamide has been shown to partially inhibit a current that is elicited by parathyroid hormone and is dependent on Cl\(^{-}\) (Laverty et al. 2003), and CFTR\(_{\text{inh}}\)172 (20 µM) has been shown to inhibit CFTR (Laverty et al. 2012) in chicken proximal tubule monolayers. Functional differences between human and chicken have been observed for CFTR single-channel currents measured in planar bilayers (Aleksandrov et al. 2012). That study
showed that the single-channel conductance and the probability of opening was higher for chicken CFTR than for human CFTR, but it is not known if or how this might affect the pharmacology of this protein. We found that 20 μM glibenclamide was sufficient to block the NO-dependent release of Cl⁻ from acidic organelles. The IC₅₀ values for glibenclamide inhibition of human CFTR are generally in the range of 20–40 μM, depending on the expression system (Sheppard and Robinson 1997; Sheppard and Welsh 1992), and in guinea pig cardiac myocytes, the value ranges from 25 to 38 μM, depending on the method of current activation (Tominaga et al. 1995). We also found that glibenclamide and CFTRαab172 are reversible, but studies on human CFTR have found that both of these inhibitors can be difficult to reverse. However, context seems to matter, because glibenclamide is easily reversible when applied to excised inside-out membrane patches of human CFTR expressed in mammary epithelial cells (Sheppard and Robinson 1997) but not for whole cell recordings from NIH 3T3 cells expressing human CFTR (Sheppard and Welsh 1992). Our recordings were made in the whole cell configurations, but we detected changes due to the functioning of chicken CFTR on intracellular membranes. It is possible that species differences and/or the intracellular (vs. plasma membrane) environments play a role in the reversibility that we observe.

Our present work demonstrates that functional CFTR is necessary for NO to increase cytosolic Cl⁻, but is it sufficient? Our previous research demonstrated that the NO-dependent release of Cl⁻ from acidic organelles depends on transient cytosolic acidification (McMains and Gleason 2011) and the maintenance of compartmental acidic environments (Krishnan and Gleason 2015). Clearly, there are other physiological factors involved, and further complexity may originate from CFTR itself.

CFTR can be regulated by multiple enzymes (Dahan et al. 2001), including protein kinase C (Jia et al. 1997), some tyrosine kinases (Billet et al. 2016), and AMP-dependent protein kinase (Hallows et al. 2000), as well as phosphatases (Luo et al. 1998). At the most fundamental level, however, CFTR activity is dependent on ATP binding and hydrolysis and is substantially enhanced by phosphorylation via protein kinase A (PKA). Under our recording conditions, AIP should be readily available; however, these experiments were done without overt activation of adenylate cyclase or addition of cAMP. One possibility is that NO triggers channel opening in the absence of PKA-dependent phosphorylation. Alternatively,
resting PKA activity levels may be relatively high in amacrine cells. Interestingly, two reports on the role of CFTR in neuronal cytosolic Cl\(^-\) homeostasis demonstrate effects of CFTR inhibitors on cytosolic Cl\(^-\) in the absence of stimulated PKA activity (Morales et al. 2011; Ostroumov et al. 2011), implying significant baseline CFTR (and possibly PKA) activity in those neurons. A third possibility is that the Ca\(^{2+}\) elevations that we know are generated by NO in amacrine cells (Maddox and Gleason 2017) activate the Ca\(^{2+}\)-sensitive adenylate cyclase, adenylate cyclase 1. CFTR activation via adenylate cyclase 1 has been previously demonstrated (Billet and Hanrahan 2013). Importantly, we have evidence that adenylate cyclase 1 is functionally expressed in amacrine cells and that there are significant levels of basal activity of both adenylate cyclase 1 and PKA in these cells (Tekmen and Gleason 2010). Further experiments examining the effects of PKA activators and inhibitors on cytosolic Cl\(^-\) and its regulation by NO would shed light on the possibility that cytosolic Cl\(^-\) and cAMP levels are linked.

CFTR has physical and physiological interactions with multiple classes of proteins. Among these are PDZ domain scaffolding proteins, which can facilitate interactions with other proteins and potentially establish signaling complexes (reviewed in Li and Naren 2010). Functional interactions have also been demonstrated for CFTR and soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins (reviewed in Tang et al. 2011). CFTR is also known to functionally interact with the renal outer medullary K\(^+\) channel ROMK, an inward rectifying K\(^+\) channel (Yoo et al. 2004), the epithelial Na\(^+\) channel ENaC (Stutts et al. 1997), Cl\(^-\)-bicarbonate exchangers (Shumaker et al. 1999), and Na\(^+\)-bicarbonate exchangers (Ko et al. 2002). Additionally, reciprocal inhibitory interactions have been demonstrated for anoctamin-1, a Ca\(^{2+}\)-activated Cl\(^-\) channel, and CFTR in epithelial cells (Ousingsawat et al. 2011), suggesting that the balance of Ca\(^{2+}\) and cAMP signaling can determine the level of Cl\(^-\) flux (Kunzelmann and Mehta 2013). Thus there is precedence for CFTR working as a part of a regulatory complex, which is consistent with the possibility that CFTR is necessary but perhaps functions in combination with other proteins in amacrine cells.

Epithelial cells employ an orchestra of transport mechanisms that by their apical or basal expression patterns regulate the distribution of ions across tissues. Neurons have a similarly complex set of transport mechanisms, but most of these regulate ion concentrations across cellular membranes and have the potential to generate nonuniform distributions of transported ions in the cytosol. Cytosolic Ca\(^{2+}\), for example, is so tightly regulated and buffered that its potential for diffusion is highly limited. The cellular scenario for Cl\(^-\) transport, regulation, and distribution is much more incomplete. Although several families of Cl\(^-\) channels and transporters have been identified (Stauber and Jentsch 2013), relatively little is known about their cellular distributions and functional interactions, especially in the context of neurons. Learning more about this will be of particular importance in understanding the function of amacrine cells, because some are capable of highly localized synaptic signaling (Grimes et al. 2010, 2015).

In this study, we have demonstrated a neuronal role for CFTR in regulating cytosolic Cl\(^-\) in response to NO, thus regulating the strength or even the sign of postsynaptic GABAergic and glycnergic responses. The involvement of CFTR in this fundamental aspect of neuronal physiology contributes to filling the gap in our understanding of neuronal Cl\(^-\) regulation. Continued investigations into the function of CFTR in the neuronal environment will likely reveal other roles for this multifunctional protein.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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