British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02041.x
www.brjpharmacol.org

RESEARCH PAPER

Pseudohalide anions reveal a novel extracellular site for potentiators to increase CFTR function

Man-Song Li, Elizabeth A Cowley and Paul Linsdell

Department of Physiology & Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada

Correspondence
Paul Linsdell, Department of Physiology & Biophysics, Dalhousie University, PO Box 15000, Halifax, Nova Scotia, Canada B3H 4R2. E-mail: paul.linsdell@dal.ca

Keywords
anion binding site; chloride channel; cystic fibrosis; cystic fibrosis transmembrane conductance regulator; potentiator; patch clamp

Received
15 August 2011
Revised
19 April 2012
Accepted
13 May 2012

BACKGROUND AND PURPOSE

There is great interest in the development of potentiator drugs to increase the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis. We tested the ability of several anions to potentiate CFTR activity by a novel mechanism.

EXPERIMENTAL APPROACH

Patch clamp recordings were used to investigate the ability of extracellular pseudohalide anions ($Co(CN)_6^{3-}$, $Co(NO_2)_6^{3-}$, $Fe(CN)_6^{3-}$, $IrCl_6^{3-}$, $Fe(CN)_6^{4-}$) to increase the macroscopic conductance of mutant CFTR in intact cells via interactions with cytoplasmic blocking anions. Mutagenesis of CFTR was used to identify a possible molecular mechanism of action. Transepithelial short-circuit current recordings from human airway epithelial cells were used to determine effects on net anion secretion.

KEY RESULTS

Extracellular pseudohalide anions were able to increase CFTR conductance in intact cells, as well as increase anion secretion in airway epithelial cells. This effect appears to reflect the interaction of these substances with a site on the extracellular face of the CFTR protein.

CONCLUSIONS AND IMPLICATIONS

Our results identify pseudohalide anions as increasing CFTR function by a previously undescribed molecular mechanism that involves an interaction with an extracellular site on the CFTR protein. Future drugs could utilize this mechanism to increase CFTR activity in cystic fibrosis, possibly in conjunction with known intracellularly-active potentiators.

Abbreviations

BHK, baby hamster kidney; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; I_{SC}, transepithelial short circuit current

Introduction

Cystic fibrosis (CF) is caused by genetic mutations that lead to loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR functions as an anion channel that is present in the apical membrane of many different epithelial cells, where it acts to control transepithelial salt and water transport (Gadsby *et al.*, 2006). Because CFTR function is low or absent in CF, it is important to understand the mechanisms controlling CFTR activity.

Physiological regulation of CFTR is predominantly by agonists that act through the cAMP/PKA pathway, leading to phosphorylation of CFTR and activation of channel function (Gadsby and Nairn, 1999; Kidd *et al.*, 2004; Alzamora *et al.*, 2011). Other intracellular factors, including other protein kinases and direct interactions with other proteins, may also influence CFTR activity (Guggino and Stanton, 2006; Li and Naren, 2010). While channel activity is usually thought of as being controlled by intracellular factors, CFTR-mediated ion transport may also be sensitive to the concentration of different anions in the extracellular solution (Wright *et al.*, 2004; Li *et al.*, 2011). Recently, we proposed that changes in extracellular anion concentrations may be a physiologically relevant factor controlling overall CFTR function in epithelial tissues (Li *et al.*, 2011). We showed that extracellular Cl^- increased CFTR channel macroscopic conductance in intact cells by reducing channel inhibition by cytoplasmic substances, suggesting that Cl^- itself acts to increase CFTR function when present in the extracellular fluid. This work suggests that cytosolic anions that produce voltage-dependent block of CFTR are physiologically relevant regulators of overall channel function, and furthermore that Cl^- can also act as a physiologically relevant regulator when present in epithelial secretions via its ability to modulate the degree of voltage-dependent block (Li *et al.*, 2011).

In the context of CF pharmacology, understanding the mechanisms controlling CFTR activity is helpful if it can teach us how to maximize CFTR function therapeutically. For example, understanding how CFTR activity is controlled by the cAMP/PKA pathway led to the suggestion that CFTR function could be increased by drugs that stimulate this pathway, such as PDE inhibitors (Becq *et al.*, 2011). However, such drugs are not specific enough to CFTR to be clinically useful in CF. More recently, drugs that directly interact with the CFTR protein to increase its channel activity – so-called 'CFTR potentiators' – have been discovered in the laboratory, and some are currently undergoing clinical trials (Verkman and Galietta, 2009; Anderson, 2010; Cuthbert, 2010; Becq *et al.*, 2011). Known potentiators increase the open probability of activated CFTR channels by interacting with an intracellular site on the protein (Moran *et al.*, 2005; Zegarra-Moran *et al.*, 2007; Huang *et al.*, 2009). Potentiators might be clinically useful in relatively infrequent cases of CF where patients retain some CFTR function, or they might be capable of acting in concert with 'correctors' that act by rescuing the defective trafficking of mutant CFTR and restoring it to the cell membrane in more common CF cases caused by protein mistrafficking. However, to our knowledge, there are no compounds known directly to stimulate CFTR activity from the extracellular side of the membrane, or even a described mechanism by which such compounds could theoretically act.

We have considered the question, if extracellular Cl^- ions act to increase CFTR function by a defined mechanism, is it possible to find pharmacological agents that mimic this effect, leading to an increase in overall CFTR-mediated anion transport? Previously, we showed that addition of the divalent pseudohalide anion, $Pt(NO_2)_4^{2-}$ (tetranitroplatinate) to the extracellular side of the membrane disrupts interactions between CFTR and intracellularly applied open channel blockers (Ge and Linsdell, 2006; Zhou and Linsdell, 2009). This suggests that $Pt(NO_2)_4^{2-}$ might be able to mimic the effect of external Cl^- ions on channel macroscopic conductance in intact cells. However, even if this were the case, we would not expect $Pt(NO_2)_4^{2-}$ to increase overall anion transport in CFTR, since $Pt(NO_2)_4^{2-}$ is also able to enter into the channel pore and block Cl^- permeation directly (Ge and Linsdell, 2006; Zhou *et al.*, 2007). This direct pore-blocking effect was apparently not shared by the trivalent anion $Fe(CN)_6^{3-}$ (ferricyanide) (Ge and Linsdell, 2006), suggesting that multivalent anions might be better able to maximize CFTR conductance. Here we tested this idea and show that a range of tri- and tetravalent pseudohalide anions increase the macroscopic conductance of a constitutively active mutant form of CFTR in intact cells under low, but not high, external Cl^- concentration conditions. These results suggest that these substances mimic the stimulating effect of external Cl^-. The effects of these compounds, as well as that of Cl^- itself, were sensitive to mutations on an extracellular-facing part of the CFTR protein, providing some information on their potential molecular mechanism of action. Furthermore, these same compounds were able to stimulate transepithelial anion secretion in CFTR-expressing human airway epithelial cells, in a manner that was additive with the effects of intracellularly acting stimulants. These results provide important proof-of-principle for the existence of an externally accessible pharmacophore that could be targeted by CFTR potentiators for the treatment of CF.

Methods

Cells used

Patch clamp experiments were carried out on baby hamster kidney (BHK) cells transiently transfected with different forms of human CFTR, as described previously (Gong *et al.*, 2002). Macroscopic current recordings were carried out in an E1371Q-CFTR background (see below). In some experiments, additional mutations (R334Q, K892Q, R899Q) were introduced into this background using the QuikChange site-directed mutagenesis system. Single channel recordings were carried out on wild-type CFTR channels. Transepithelial short-circuit current recordings were carried out on confluent polarized monolayers of Calu-3 human airway epithelial cells grown at an air–liquid interface as described in detail previously (Cowley and Linsdell, 2002).

Patch clamp recording of CFTR

In order to monitor and compare CFTR macroscopic conductance in intact cells and in excised membrane patches, we used mutant E1371Q-CFTR channels that show constitutive, high levels of activity when expressed in BHK cells (Zhou *et al.*, 2010; Li *et al.*, 2011). Use of this mutant channel is necessary for experimental separation of effects on channel conductance and channel gating (Li *et al.*, 2011). Patch clamp recording of E1371Q–CFTR macroscopic currents was carried out as described in detail recently (Zhou *et al.*, 2010; Li *et al.*, 2011). Briefly, currents were recorded from cell-attached membrane patches following stabilization of seal resistance. Membrane patches were then excised to the inside-out configuration, and currents were recorded for several minutes to

ensure stability of current amplitude. At the end of the experiment, background (leak) currents were determined by addition of a high concentration (20 μM) of the specific CFTR inhibitor $CFTR_{inh}$-172 to the cytoplasmic solution, as described previously (Zhou *et al.*, 2010; Li *et al.*, 2011). Macroscopic current–voltage (*I–V*) relationships were constructed using depolarizing voltage ramp protocols (Linsdell and Hanrahan, 1998; Li *et al.*, 2011). Background (leak) currents recorded in the presence of $CFTR_{inh}$-172 have been digitally subtracted from all *I–V* relationships (Li *et al.*, 2011). Differences in the amplitude of leak-subtracted currents in cell-attached and inside-out patches are taken to reflect the degree of current inhibition by endogenous cytoplasmic substances in intact cells (Zhou *et al.*, 2010; Li *et al.*, 2011).

Single channel currents carried by wild-type CFTR were recorded from inside-out membrane patches following channel activation by addition of PKA catalytic subunit (5 nM) and MgATP (1 mM) to the cytoplasmic solution.

For both macroscopic and single channel current recording, the bath solution (intracellular during inside-out patch experiments) contained (mM) 150 NaCl, 2 $MgCl_2$, 10 *N*-tris[hydroxmethyl]methyl-2-aminoethanesulphonate (TES). The pipette (extracellular) solution contained the same solution (high Cl^- concentration, 154 mM), or with NaCl replaced by 150 mM Na gluconate (low Cl^- concentration, 4 mM). Different pseudohalide salts [$(K_2Pt(NO_2)_4$ (tetranitroplatinate), $Na_3Co(CN)_6$ (cobalticyanide), $Na_3Co(NO_2)_6$ (hexanitrocobaltate), $K_3Fe(CN)_6$ (ferricyanide), Na_3IrCl_6 (hexachloroiridate), $Na_4Fe(CN)_6$ (ferrocyanide)] were added to the pipette solution at the concentrations required at the beginning of the experiment. The bath solution also contained 1 mM MgATP to maintain CFTR channel activity. All solutions for patch clamp experiments were adjusted to pH 7.4 using NaOH. Solution osmolarities, as measured using a freezing point osmometer, were 324 ± 1 mOsm (high Cl^- concentration) and 314 ± 2 mOsm (low Cl^- concentration) in the absence of pseudohalides, and averaged between 321 and 344 mOsm in the presence of different concentrations of pseudohalides used (n = 3–4). Membrane voltages have been corrected for liquid junction potentials calculated using pCLAMP9 software (Molecular Devices, Sunnyvale, CA). Membrane currents were filtered at 50 Hz (for single channel currents) or 100 Hz (for macroscopic currents) using an eight-pole Bessel filter, digitized at 250 Hz (single channel currents) or 1 kHz (macroscopic currents), and recorded using pCLAMP software. Patch clamp experiments were carried out at room temperature, 21–24°C.

Transepithelial current recording

Transepithelial short-circuit current (I_{SC}) was recorded from polarized Calu-3 cell monolayers mounted in an Ussing chamber with the transepithelial potential difference clamped to zero, as described in detail previously (Cowley and Linsdell, 2002; Moser *et al.*, 2008). Apical and basolateral solutions were maintained at 37°C by heated water jackets and separately perfused and oxygenated with a 95% O_2:5% CO_2 mixture. Bath solutions contained (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2HPO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose (basolateral) or mannitol (apical), pH 7.4 at 37°C when gassed with 95% O_2:5% CO_2. Basal I_{SC} under these conditions is almost exclusively due to transepithelial anion secretion via CFTR channels in the apical membrane; this was confirmed by sensitivity to the CFTR inhibitor GlyH-101 (Figure 9). Different pseudohalide anions (sodium salts only) were added directly to the apical solution during recordings at the concentration required from high concentration stocks. Forskolin and genistein were added apically at maximally effective concentrations of 5 and 50 μM, respectively. These maximally effective concentrations, determined in preliminary experiments as concentrations at which further addition of the same stimulant was without noticeable effect, are in line with previous investigations using Calu-3 cells (e.g. Shen *et al.*, 1994; Illek and Fischer, 1998; Cowley and Linsdell, 2002). Solution osmolarities were 317 ± 2 mOsm (apical) and 315 ± 1 mOsm (basolateral) in the absence of pseudohalides and averaged between 316 and 344 mOsm in the presence of different concentrations of pseudohalides used (n = 3).

Nomenclature

The nomenclature in this paper conforms to the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al.*, 2011 9).

Chemical reagents

PKA was obtained from Promega (Madison, WI). $K_2Pt(NO_2)_4$, $Na_3Co(NO_2)_6$ and Na_3IrCl_6 were from Strem Chemicals (Newburyport, MA). $Na_3Co(CN)_6$ and $Na_4Fe(CN)_6$ were from City Chemical (West Haven, CT). GlyH-101 was from EMD Chemicals (Gibbstown, NJ). All other chemicals were from Sigma-Aldrich (Oakville, ON, Canada). QuikChange site directed mutagenesis system was from Agilent Technologies (Santa Clara, CA).

Results

Stimulation of conductance by extracellular pseudohalide ions

CFTR channel currents in intact cells are subject to voltage-dependent block by endogenous cytosolic anions, leading to outward rectification of the *I–V* relationship (Zhou *et al.*, 2001; Li *et al.*, 2011). Modulation of the strength of this cytoplasmic block by extracellular anions – including Cl^- – is the basis of the regulation of CFTR macroscopic conductance by such extracellular anions (Li *et al.*, 2011). Physiologically, this may allow CFTR function to be modulated by the Cl^- content of epithelial secretions (Li *et al.*, 2011). Experimentally, these regulatory effects of extracellular anions can be monitored and quantified using the constitutively active E1371Q–CFTR mutant, as described in detail previously (Li *et al.*, 2011). With low extracellular Cl^- concentrations, E1371Q–CFTR currents in intact cells are small due to block by cytosolic anions, the extent of which can be observed by the increase in current amplitude due to relief of block when the membrane patch is excised to the inside-out configuration (Li *et al.*, 2011). This effect is shown in Figure 1 (4 mM extracellular Cl^-). We reasoned that if extracellular pseudohalide anions mimicked the effect of Cl^- in relieving channel block in intact cells, then inclusion of these anions in the pipette solution would increase current amplitude in cell-

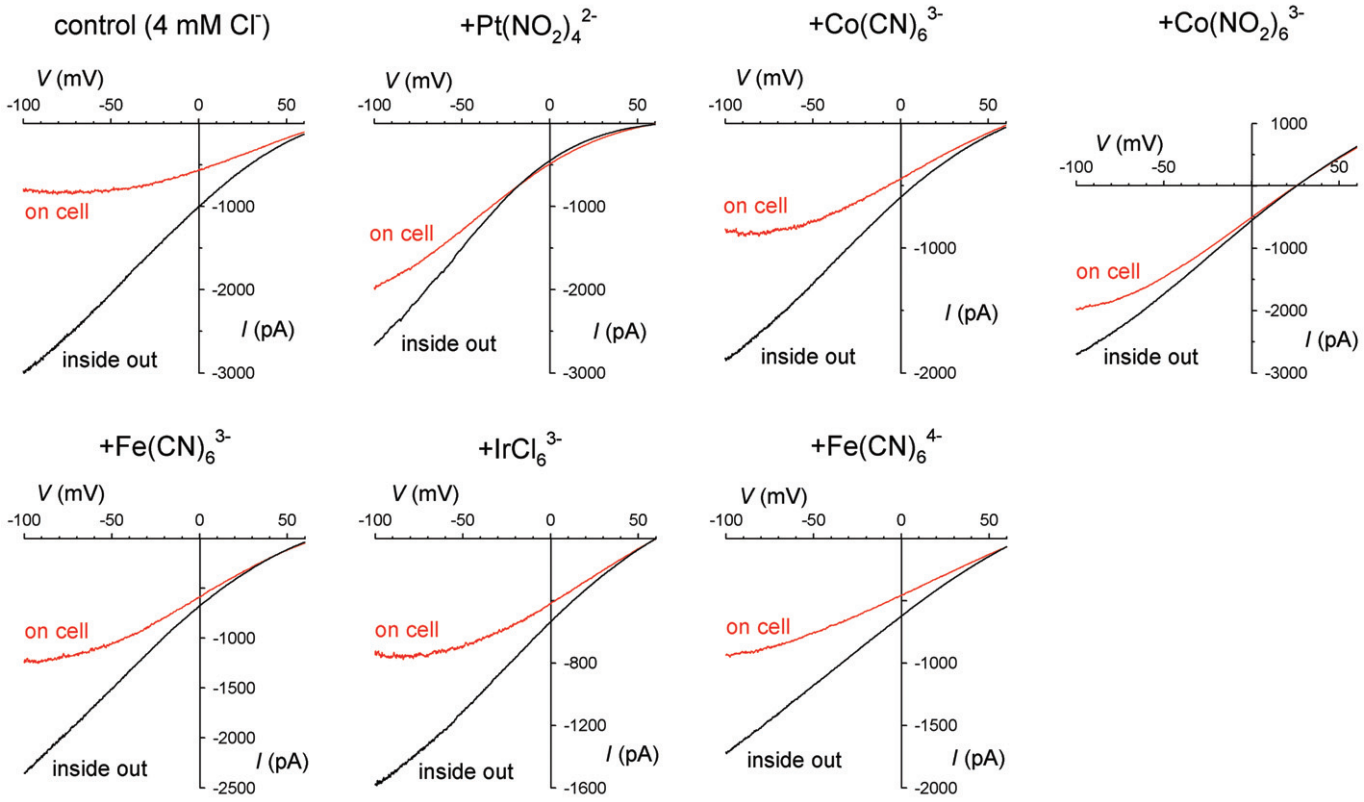

Figure 1

Effect of external pseudohalide anions on macroscopic E1371Q–CFTR currents in cell-attached and inside-out membrane patches recorded with low extracellular chloride concentration. Example leak-subtracted macroscopic *I–V* relationships for E1371Q–CFTR recorded under low (4 mM) extracellular Cl^- concentration conditions. Constitutively active currents were recorded from cell-attached patches (on cell) and immediately after patch excision (inside out). The six different named pseudohalide anions were included in the extracellular (pipette) solution at a concentration of 10 mM. Note the current reversal and appearance of outward currents in the presence of $Co(NO_2)_6^{3-}$, consistent with this anion carrying some current in CFTR.

attached patches, leading to a reduction in the magnitude of the increase in current amplitude observed after patch excision. Figure 1 shows example macroscopic *I–V* relationships recorded under these conditions with six different pseudohalide anions present in the pipette solution – the divalent $Pt(NO_2)_4^{2-}$, the trivalent $Co(CN)_6^{3-}$, $Co(NO_2)_6^{3-}$, $Fe(CN)_6^{3-}$ and $IrCl_6^{3-}$, and the tetravalent $Fe(CN)_6^{4-}$ (each at 10 mM). As shown in Figure 2, each of these anions significantly reduced the degree of current inhibition seen in intact cells, as quantified as the current amplitude during cell-attached patch recording as a fraction of that immediately after patch excision to the inside-out configuration. The relative potency of these anions in apparently stimulating CFTR conductance at –100 mV (where block by cytosolic anions is strongest) is summarized in Figure 3A. As shown in Figure 3B, the stimulating effects of one anion, $Co(CN)_6^{3-}$, were concentration-dependent and statistically significant only at high concentrations (10 mM). The results shown in Figures 2 and 3 suggest that each of the six pseudohalide anions tested are able to mimic the stimulating effects of external Cl^- ions on CFTR conductance via interactions with cytosolic blocking anions.

Extracellular gluconate ions are not permeant in CFTR (Linsdell and Hanrahan, 1998), and as such, the macroscopic currents recorded in Figure 1 are expected to reverse close to the Cl^- ion equilibrium potential of +93 mV. Consistent with this, under most conditions no current reversal was observed over the voltage range examined (–100 to +60 mV). However, when 10 mM $Co(NO_2)_6^{3-}$ was included in the extracellular solution, the current reversal potential was +26.6 ± 0.5 mV (n = 7) (see Figure 1). The simplest explanation for this finding is that $Co(NO_2)_6^{3-}$ itself is permeant and carries current through the CFTR channel. However, as it is generally assumed that the Goldman–Hodgkin–Katz equation used to calculate ionic permeabilities is not applicable to trivalent ions (Hals *et al.*, 1989), we are not able to estimate the actual permeability of $Co(NO_2)_6^{3-}$. Previously, it was shown that $Pt(NO_2)_4^{2-}$ is impermeant in CFTR (Gong and Linsdell, 2003a).

We next considered if these stimulating effects of extracellular pseudohalide anions would be additive with those of Cl^- itself. As shown quantitatively in Figure 4, only $Pt(NO_2)_4^{2-}$ and $Co(NO_2)_6^{3-}$ (at concentrations of 10 mM) were still able to significantly stimulate CFTR current amplitudes in cell-attached patches under these conditions. With most pseudohalides present in the pipette solution, currents reversed very close to the expected Cl^- equilibrium potential of 0 mV. However, with 10 mM $Co(NO_2)_6^{3-}$ in the extracellular

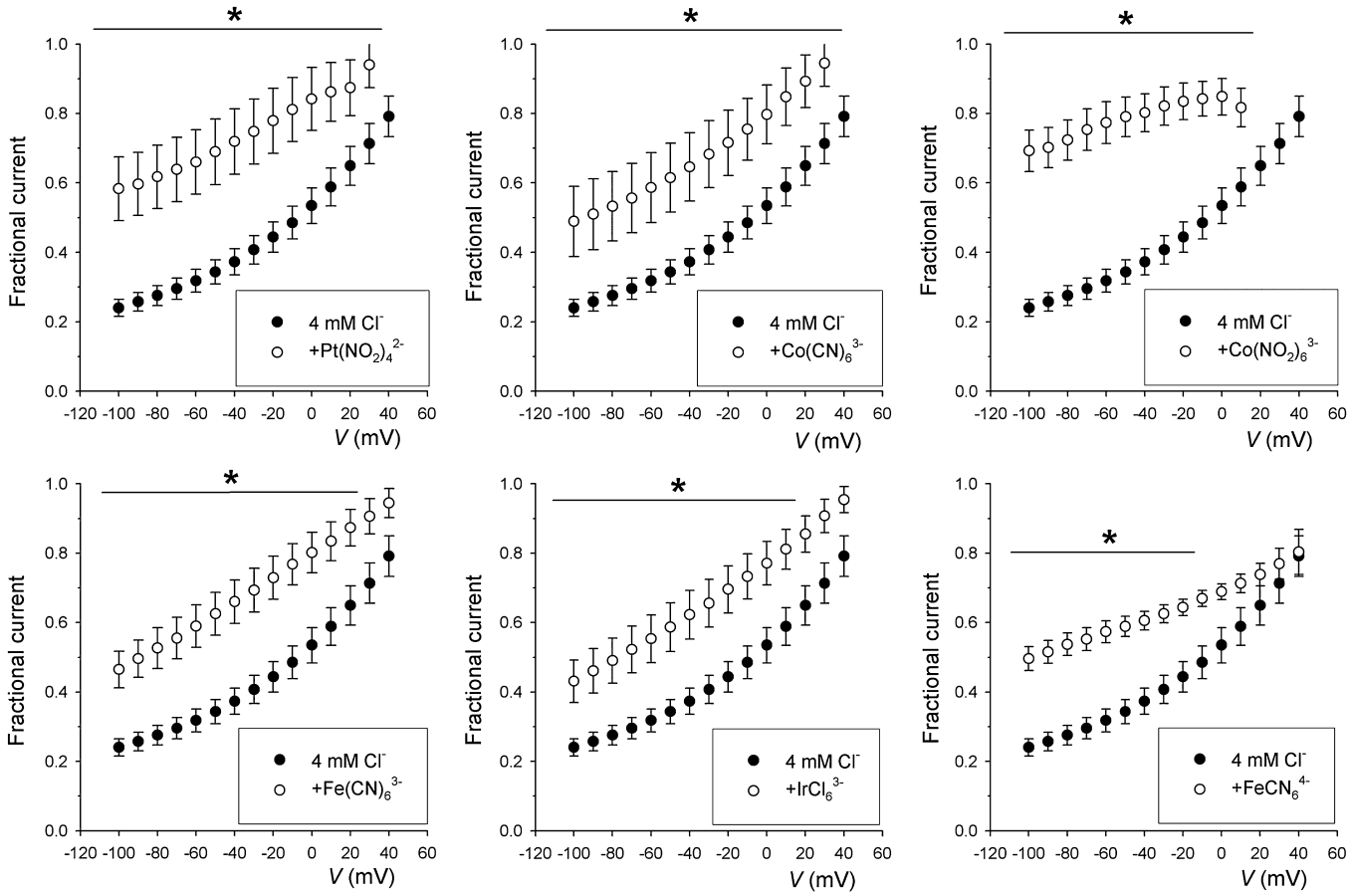

Figure 2

External pseudohalide anions weaken the apparent blocking effect of cytosolic anions under low extracellular chloride concentration conditions. The strength of channel block by cytosolic anions was quantified by measuring the macroscopic current amplitude in cell-attached patches as a fraction of current in the same membrane patch immediately after excision to the inside-out configuration. In each panel, currents recorded with the named pseudohalide anion are compared with those measured under control conditions. Asterisks indicate the voltage range over which there was a significant difference between the two conditions ($P < 0.05$). Mean of data from 5–18 patches.

solution, the measured current reversal potential was –7.1 ± 0.4 mV ($n = 4$) (Figure 4A), again consistent with some degree of $Co(NO_2)_6^{3-}$ permeation through the channel.

Role of positively charged amino acids in pseudohalide effects

The effects of extracellular pseudohalide anions suggest that they are able to destabilize interactions between cytosolic blocking substances and the CFTR channel. Different mechanisms have been proposed by which extracellular anions might be able to affect interactions between the channel and intracellular blocking ions. For example, extracellular anions may enter into the pore to interact electrostatically with intracellular blockers (Linsdell *et al.*, 1997; Gong and Linsdell, 2003b), or they may interact with a site outside of the pore to alter the structure of the intracellular blocker binding site inside the pore by a long-range allosteric mechanism (Ge and Linsdell, 2006; Zhou and Linsdell, 2009). Both of these potential mechanisms might involve interactions between extracellular anions and positively charged amino acids on the CFTR protein. Thus, removal (by mutagenesis) of the positive charge at R334 (in the sixth membrane spanning region of the protein) has been shown to disrupt interactions with anions inside, but not outside the pore (Zhou *et al.*, 2007); while removal of charges in the fourth extracellular loop (K892, R899) has been shown to disrupt interactions with anions outside, but not inside the pore (Zhou and Linsdell, 2009). To gain information on the molecular mechanism of action of extracellular pseudohalide anions, we tested their ability to stimulate CFTR conductance in E1371Q–CFTR channels in which these positively charged residues had been neutralized. We tested the effects of two pseudohalides, $Co(CN)_6^{3-}$ and $Co(NO_2)_6^{3-}$, under the low external [Cl^-] conditions where they had been found to be effective at relieving channel block in intact cells (see Figures 1–3). Examples of the macroscopic *I–V* relationships for R334Q, K892Q, R899Q and K892Q/R899Q (all in an E1371Q background) are shown in Figure 5A. Apparent block in intact cells was weak in R334Q and was not significantly weakened further by inclusion of 10 mM $Co(CN)_6^{3-}$ (Figure 5D) or $Co(NO_2)_6^{3-}$ (Figure 5E) in the pipette solution. Block of R899Q in intact cells was not significantly affected by extracellular $Co(CN)_6^{3-}$

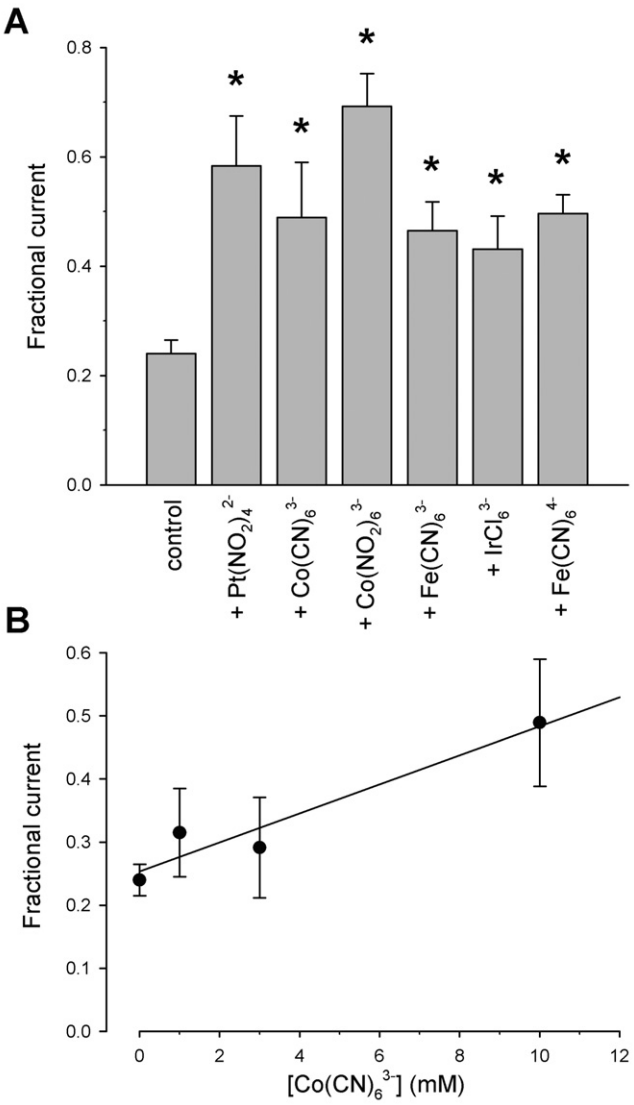

Figure 3

Relative effectiveness of different pseudohalide anions in stimulating CFTR conductance under low extracellular chloride concentration conditions. (A) Mean fractional current recorded in cell-attached patches relative to inside-out patches at a membrane potential of –100 mV under the ionic conditions used in Figure 2. Asterisks indicate a significant difference from control conditions ($P < 0.05$). (B) Concentration-dependence of $Co(CN)_6^{3-}$ effects under these conditions. Mean of data from 5–18 patches in both panels.

(Figure 5B,D) or $Co(NO_2)_6^{3-}$ (Figure 5C,E). Block of K892Q was significantly strengthened by $Co(CN)_6^{3-}$ (Figure 5D) but was unaffected by $Co(NO_2)_6^{3-}$ (Figure 5E). Neutralization of both of these extracellular positive charges, in the K892Q/R899Q/E1371Q triple mutant, resulted in apparent blocker sensitivity that, as in R899Q/E1371Q, was not significantly affected by extracellular $Co(CN)_6^{3-}$ or $Co(NO_2)_6^{3-}$ (Figure 5D,E). Each of these mutants (R334Q, K892Q, R899Q, K892Q/R899Q) also abolished the sensitivity of current inhibition in intact cells to extracellular Cl^- ions (Figure 6). The effect of neutralizing these positive charges on interactions with both pseudohalides and Cl^- ions are summarized in Figure 7.

Extracellular pseudohalide effects on Cl^- permeation

While $Pt(NO_2)_4^{2-}$ is effective at mimicking the effect of extracellular Cl^- and relieving channel block by cytosolic anions (Figures 3, 4) – consistent with earlier findings obtained by applying blockers directly to the cytosolic face of inside-out membrane patches (Ge and Linsdell, 2006; Zhou and Linsdell, 2009) – it would not be expected to increase overall CFTR function because it is also able to enter into the permeation pathway and directly block Cl^- permeation (Ge and Linsdell, 2006; Zhou *et al.*, 2007). To test if tri- or tetravalent pseudohalide anions can also enter the pore to block Cl^- permeation, we carried out single channel current recordings with these anions present in the pipette solution (at 10 mM in all cases). In order to maximize sensitivity to any such blocking effects, these experiments were carried out using a low extracellular Cl^- concentration (since external Cl^- may compete with other anions for entry into the pore). As shown in Figure 8, unitary Cl^- current amplitudes were significantly reduced when $Co(NO_2)_6^{3-}$ was present in the extracellular solution. This inhibitory effect was more pronounced at more depolarized membrane potentials that would tend to favour entry of the anion into the channel pore from the extracellular solution. This effect is therefore consistent with evidence that $Co(NO_2)_6^{3-}$ is permeant and able to enter into the channel pore (see above). However, other trivalent anions ($Co(CN)_6^{3-}$, $Fe(CN)_6^{3-}$, $IrCl_6^{3-}$) had only very small effects on Cl^- current amplitude at this 10 mM concentration that were significant only at the most depolarized voltages studied, and the tetravalent $Fe(CN)_6^{4-}$ was without significant effect (Figure 8B). At 0 mV membrane potential, only $Co(NO_2)_6^{3-}$ and $Co(CN)_6^{3-}$ significantly affected Cl^- current amplitude at concentrations of 10 mM (Figure 8C).

Effects on CFTR-dependent epithelial anion secretion

The ability of multivalent pseudohalide anions to increase CFTR open channel conductance in intact cells without strongly blocking Cl^- permeation suggests they might act to increase overall CFTR function. To test the ability of such anions to increase CFTR function in a more physiological epithelial cell system, we investigated the effect of several anions on CFTR-dependent anion secretion by polarized monolayers of Calu-3 human airway epithelial cells (Figure 9). Basal I_{SC} in intact Calu-3 cell monolayers, which reflects CFTR-dependent anion secretion (Singh *et al.*, 1997; Cowley and Linsdell, 2002), was increased by the addition of $Co(CN)_6^{3-}$ to the apical face of the monolayer in a concentration-dependent fashion (Figure 9A, C). The short-circuit current under these conditions was inhibited by the specific CFTR blocker GlyH-101 (Figure 9A), confirming the requirement for CFTR activity for this response. Furthermore, pretreatment with GlyH-101 not only reduced basal I_{SC} but also significantly attenuated the effects of subsequent addition of $Co(CN)_6^{3-}$ (Figure 9B, D). High concentrations (10 mM) of $Co(NO_2)_6^{3-}$, $IrCl_6^{3-}$ and $Fe(CN)_6^{4-}$ also increased basal I_{SC} (Figure 9D). These stimulating effects on I_{SC} support

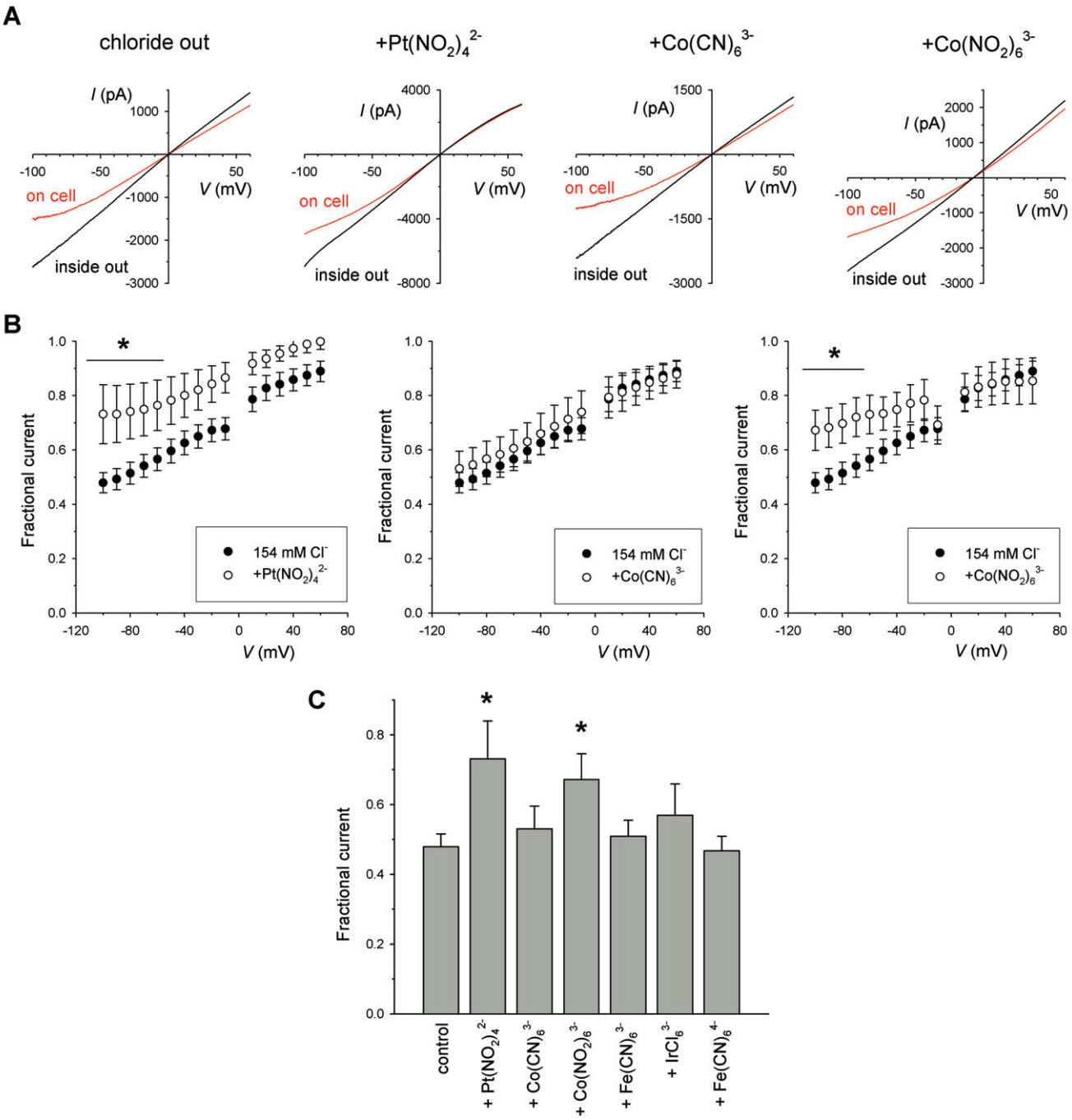

Figure 4

Effect of external pseudohalide anions on the apparent blocking effect of cytosolic anions under high extracellular chloride concentration conditions. (A) Example leak-subtracted macroscopic *I–V* relationships for E1371Q–CFTR recorded under high (154 mM) extracellular Cl^- concentration conditions. Constitutively active currents were recorded from cell-attached patches (on cell) and immediately after patch excision (inside out). The three different named pseudohalide anions were included in the extracellular (pipette) solution at a concentration of 10 mM. Note the slight leftward (hyperpolarizing) shift in current reversal potential in the presence of $Co(NO_2)_4^{3-}$, again consistent with this anion carrying some current in CFTR. (B) The strength of channel block by cytosolic anions was quantified by measuring the macroscopic current amplitude in cell-attached patches as a fraction of current in the same membrane patch immediately after excision to the inside-out configuration. In each panel, currents recorded with the named pseudohalide anion are compared with those measured under control conditions. Asterisks indicate the voltage range over which there was a significant difference between the two conditions ($P < 0.05$). Other pseudohalide anions tested, like $Co(CN)_6^{3-}$, had no significant effect across the entire voltage range studied (not shown). (C) Mean fractional current recorded in cell-attached patches relative to inside-out patches at a membrane potential of –100 mV under the ionic conditions used in (A) and (B). Asterisks indicate a significant difference from control conditions ($P < 0.05$). Mean of data from 5–15 patches.

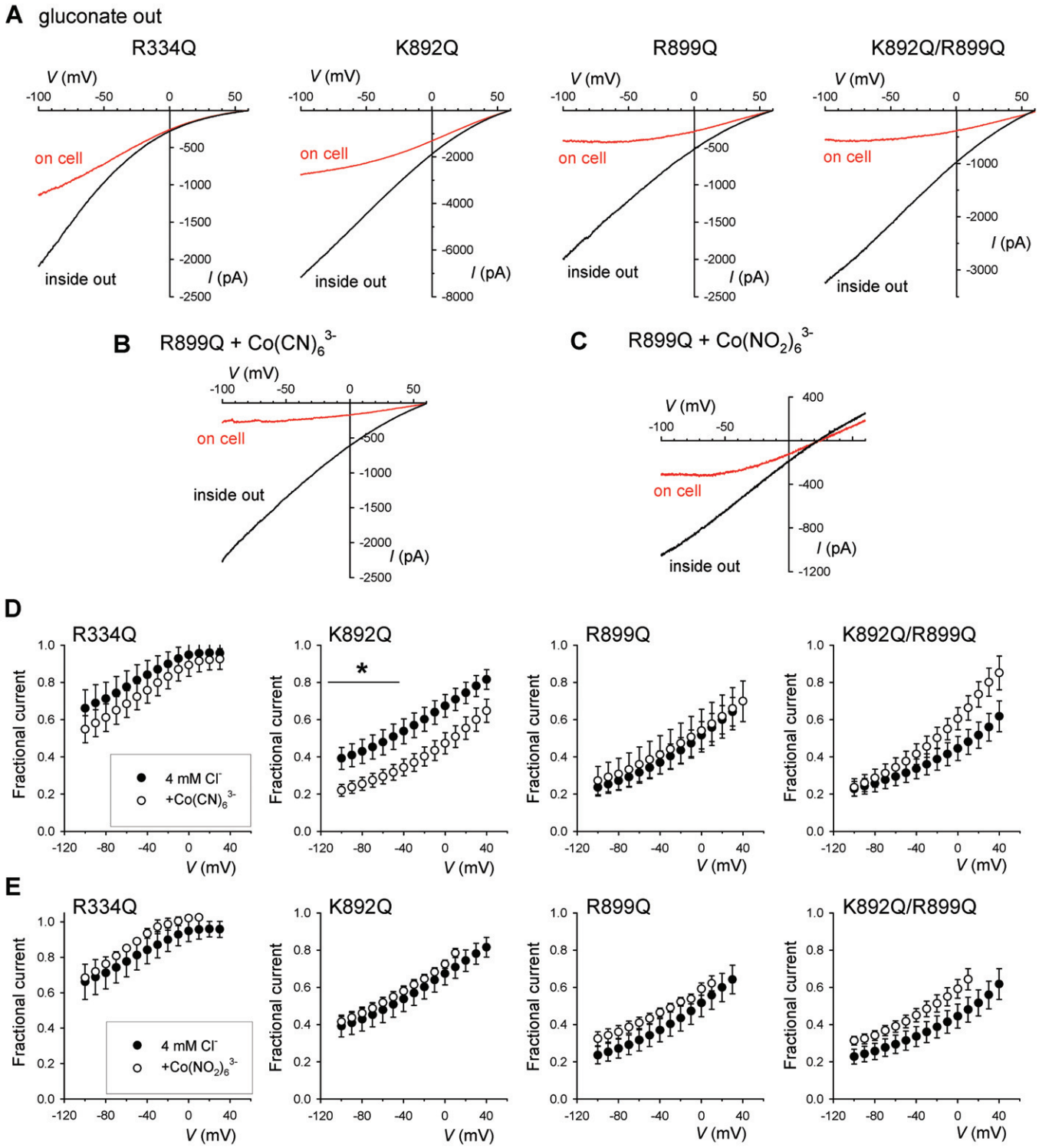

Figure 5

Effect of mutating positively charged amino acids on the effectiveness of extracellular pseudohalide anions in stimulating CFTR conductance in cell-attached patches. (A–C) Examples of leak-subtracted macroscopic *I–V* relationships for different mutants in an E1371Q–CFTR background recorded under low (4 mM) extracellular Cl^- concentration conditions (compare E1371Q alone in Figure 1). Constitutively active currents were recorded from cell-attached patches (on cell) and immediately after patch excision (inside out). (A) No pseudohalide anion; (B) 10 mM extracellular $Co(CN)_6^{3-}$; (C) 10 mM extracellular $Co(NO_2)_6^{3-}$. (D, E) Mean fractional current recorded in cell-attached patches relative to inside-out patches for each of these mutants, under control conditions or in the presence of extracellular $Co(CN)_6^{3-}$ (D) or $Co(NO_2)_6^{3-}$ (E) as indicated. Asterisks indicate the voltage range over which there was a significant difference between the two conditions ($P < 0.05$). Mean of data from 3–10 patches.

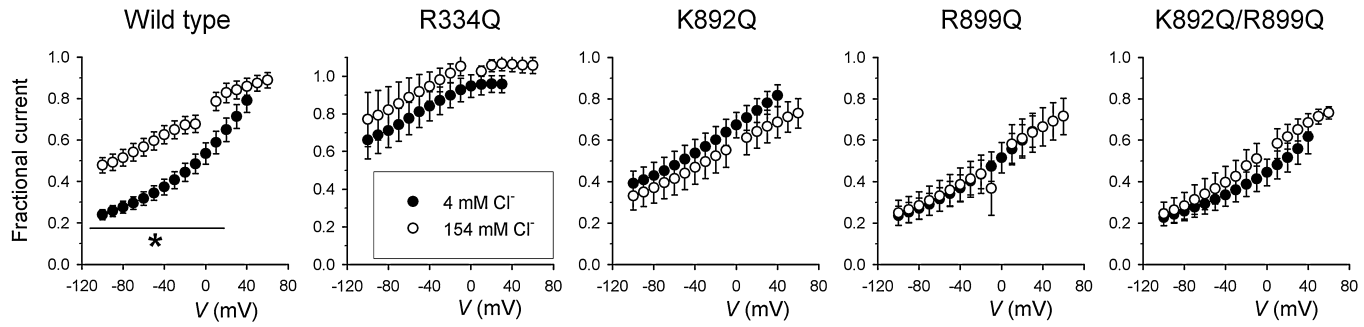

Figure 6

Mutating positively charged amino acids removes the effect of extracellular Cl^- on CFTR conductance in cell-attached patches. Mean fractional current recorded in cell-attached patches relative to inside-out patches for each of the channel variants named (in each case, in an E1371Q background), under low (4 mM) or high (154 mM) extracellular Cl^- concentration conditions. Asterisks indicate the voltage range over which there was a significant difference between the two conditions ($P < 0.05$). Mean of data from 3–10 patches.

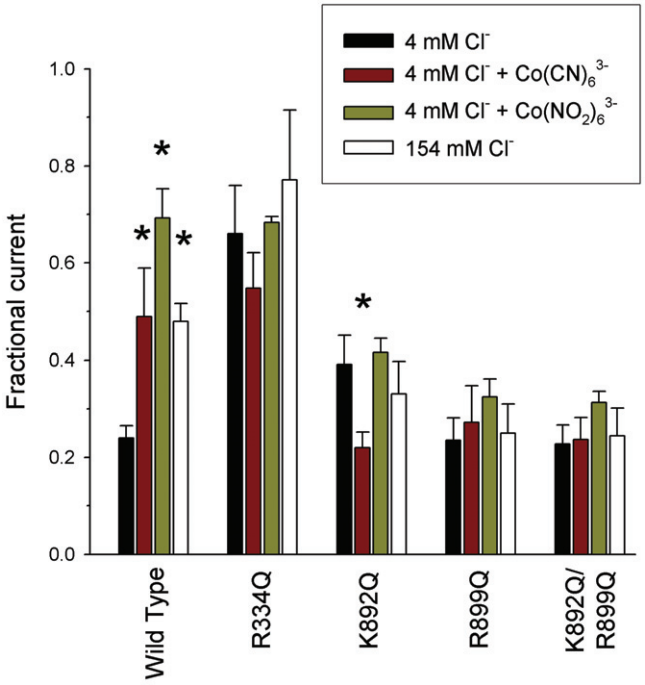

Figure 7

Extracellular anion sensitivity of macroscopic conductance in cell-attached patches in mutant forms of CFTR. Mean fractional current recorded in cell-attached patches relative to inside-out patches at a membrane potential of –100 mV under the ionic conditions used in Figures 5 and 6. Asterisks indicate a significant difference from low (4 mM) extracellular Cl^- concentration conditions ($P < 0.05$). Mean of data from 3–10 patches.

the idea that these compounds can act from the extracellular solution to increase CFTR-dependent processes in human epithelial cells.

The stimulating effects of $Co(CN)_6^{3-}$ on I_{SC} were relatively small compared with those of forskolin, a stimulant of the intracellular cAMP pathway, or genistein, an intracellular-acting CFTR potentiator (Figure 10). However, even following maximal stimulation of I_{SC} by these substances (see Methods), $Co(CN)_6^{3-}$ was still able to evoke a further increase in I_{SC}, consistent with this pseudohalide acting independently of intracellular stimulating pathways (Figure 10). In fact, the effect of $Co(CN)_6^{3-}$ on I_{SC} was significantly greater in Calu-3 cell monolayers that had already been stimulated with forskolin (Figure 10).

Discussion

There is much interest in the development of potentiators of CFTR function as potential therapeutics in CF (Verkman and Galietta, 2009; Anderson, 2010; Cuthbert, 2010; Becq *et al.*, 2011). Known potentiators are likely to act by increasing the open probability of activated CFTR channels via action at a cytoplasmic site on the protein, favouring the open state of the channel (Moran *et al.*, 2005; Zegarra-Moran *et al.*, 2007; Huang *et al.*, 2009). Our work provides the first evidence that non-physiological substances can interact with a site on the extracellular side of the CFTR protein itself to increase its function. Evidence that multivalent pseudohalide anions can increase CFTR function comes from the ability of these compounds to increase apparent macroscopic conductance of the constitutively active E1371Q-CFTR mutant in intact cells. Previously, we showed that extracellular Cl^- has a similar stimulating effect on E1371Q-CFTR conductance in intact cells, via its ability to destabilize the blockage of open channels by cytosolic substances, blockage which acts to decrease CFTR conductance in a voltage-dependent fashion (Li *et al.*, 2011). Consistent with this mechanism of action, the effects of most pseudohalide anions were apparently not additive with those of Cl^-, since these anions were effective under low (Figures 2, 3) but not high (Figure 4) extracellular Cl^- concentration conditions. While we suggested that the effect of extracellular Cl^- on the conductance of open CFTR channels might be physiologically relevant in Cl^--secretory epithelia (Li *et al.*, 2011), our present results using multivalent pseudohalide anions suggest that this effect also has the potential to be manipulated pharmacologically. In support of this idea, several pseudohalides were able to increase anion

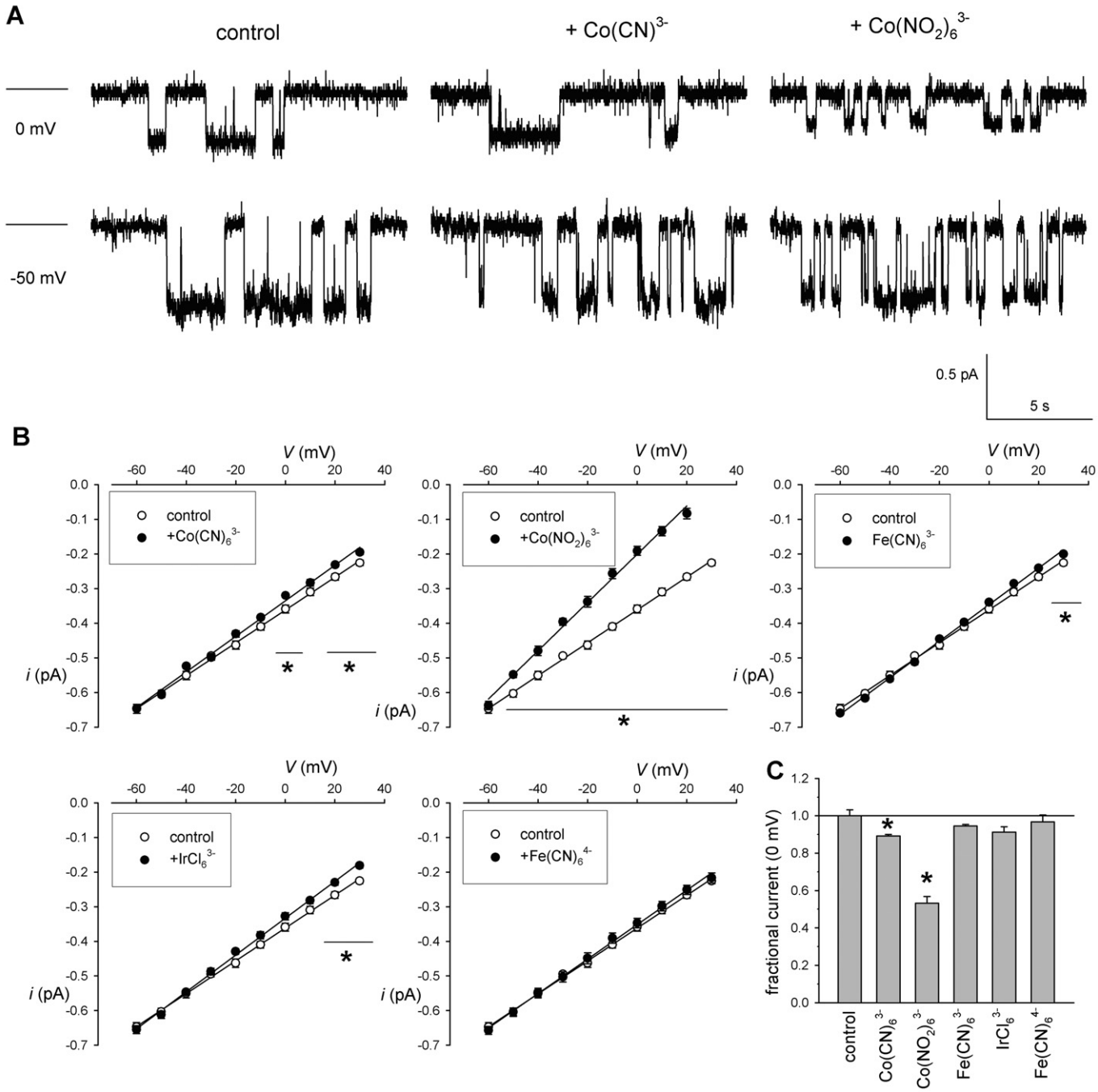

Figure 8

Effect of external pseudohalide anions on unitary Cl^- current amplitude. (A) Example single channel currents carried by wild-type CFTR recorded under low (4 mM) extracellular Cl^- concentration conditions, at membrane potentials of 0 and −50 mV as indicated on the left. Line to the left indicates the closed channel current level. At each membrane potential, currents were recorded in the absence of pseudohalide (control) or with 10 mM $Co(CN)_6^{3-}$ or $Co(NO_2)_6^{3-}$ in the pipette solution as indicated. (B) Mean single *I–V* relationships under these conditions for control and in the presence of 10 mM of the named psuedohalide anion. Asterisks indicate the voltage range over which there was a significant difference between the two conditions ($P < 0.05$). (C) Comparison of the effects of different pseudohalide anions on unitary current amplitude at a membrane potential of 0 mV. Asterisks indicate a significant difference from control ($P < 0.05$). Mean of data from 4–7 patches in (B) and (C).

secretion by polarized Calu-3 cell monolayers in a CFTR-dependent fashion when applied to the apical membrane (Figure 9). Currently, the mechanism by which these compounds stimulate anion secretion in Calu-3 cell monolayers, and its relationship to their effect on E1371Q-CFTR macroscopic conductance in BHK cells, are not known. Indeed, the effects of these anions on Calu-3 cell anion secretion were observed under high extracellular Cl^- conditions (~125 mM),

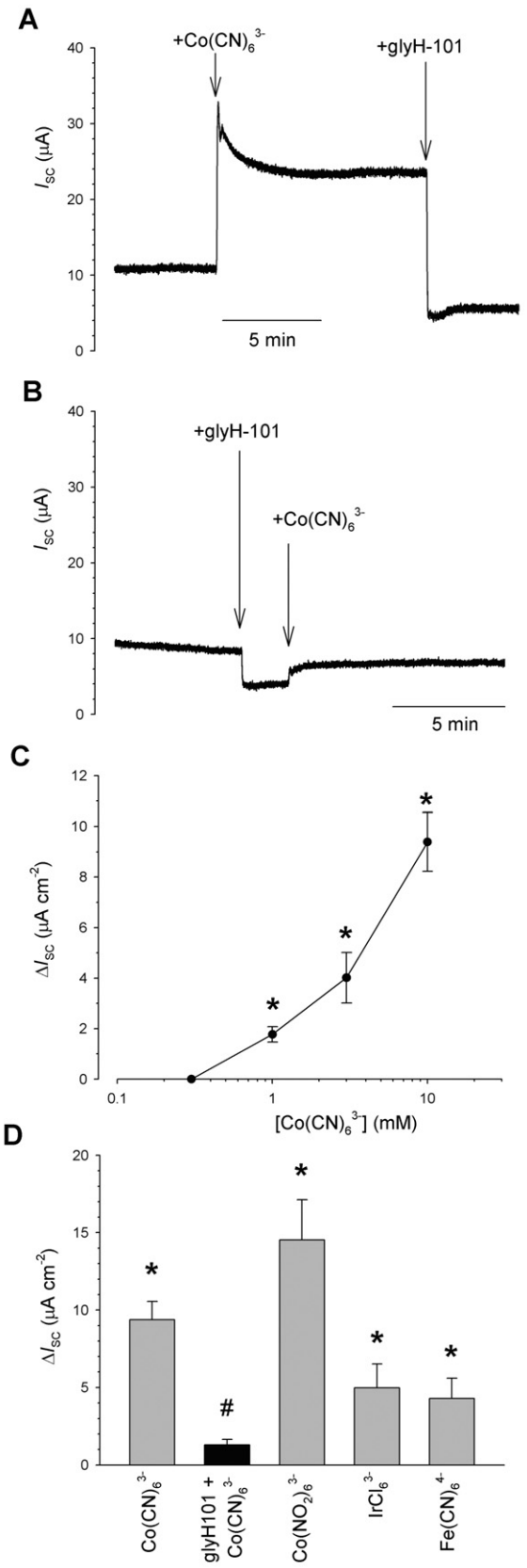

Figure 9

Effect of external pseudohalide anions on anion secretion in polarized Calu-3 cell monolayers. (A) Example of the increase in I_{SC} in response to addition of 10 mM $Co(CN)_6^{3-}$ to the apical face of a Calu-3 cell monolayer. Overall I_{SC} was almost completely blocked by addition of the CFTR-specific inhibitor glyH-101 (50 μM) to the apical solution. (B) Example of a recording showing that application of this concentration of glyH-101 blocked basal I_{SC}, and under these conditions addition of 10 mM $Co(CN)_6^{3-}$ resulted in only a very minor increase in I_{SC}. (C) Mean change in I_{SC} following addition of different concentrations of $Co(CN)_6^{3-}$ to the apical face of monolayers. ΔI_{SC} was measured for the sustained component of the response to addition of $Co(CN)_6^{3-}$. (D) Columns indicate the mean change in I_{SC} following addition of the different pseudohalide anions indicated (at a concentration of 10 mM) to the apical face of monolayers. Asterisks indicate a significant difference from control ($P < 0.05$). Solid column indicates the response to 10 mM $Co(CN)_6^{3-}$ following pretreatment with 50 μM glyH-101. # Indicates a significant difference from 10 mM $Co(CN)_6^{3-}$ alone ($P < 0.001$). Mean of data from 3–9 monolayers in (C) and (D).

suggesting they might have effects on these epithelial cells unrelated to their effects on E1371Q-CFTR channels. However, $Co(CN)_6^{3-}$ was still able to stimulate anion secretion following maximal stimulation by either forskolin or genistein (Figure 10), suggesting that it does not act through the intracellular pathways targeted by these compounds. Alternatively, it is possible that the susceptibility of CFTR in these cells to cytosolic block, and consequently the sensitivity of overall CFTR conductance to extracellular anions that can modulate block, is different in these epithelial cells compared to CFTR-overexpressing BHK cells. In any event, the stimulating effects on anion secretion in Calu-3 cells offer encouragement that CFTR-dependent processes can be stimulated by substances acting at an extracellular site.

Extracellular $Pt(NO_2)_4^{2-}$ ions are able to enter into the CFTR channel pore, leading to a kinetically fast, strongly voltage-dependent block of Cl^- permeation (Ge and Linsdell, 2006; Zhou *et al.*, 2007). In contrast, $Co(CN)_6^{3-}$, $Fe(CN)_6^{3-}$, $IrCl_6^{3-}$ and $Fe(CN)_6^{4-}$ had minimal effects on Cl^- conductance when present in the extracellular solution at concentrations of 10 mM (Figure 8), suggesting that they show a very low ability to enter the pore. Previously, it was suggested that the ability of lyotropic anions to enter the channel pore from the intracellular solution was charge-dependent (favouring lower valency) (Gong and Linsdell, 2003a), and the results shown in Figure 8 support a similar charge dependence for anion entry into the pore from the extracellular solution. However, one trivalent anion, $Co(NO_2)_6^{3-}$, showed a much greater ability to enter the pore – not only did this anion affect unitary Cl^- current amplitude when present in the extracellular solution at a concentration of 10 mM (Figure 8), but surprisingly $Co(NO_2)_6^{3-}$ appeared able to permeate through the channel (Figure 1). While extracellular $Pt(NO_2)_4^{2-}$ blocks Cl^- permeation in CFTR, suggesting that it can enter the pore (Ge and Linsdell, 2006), it is not measurably permeant (Gong and Linsdell, 2003a). The specific properties of $Co(NO_2)_6^{3-}$ that enable it to pass through the channel pore are not clear;

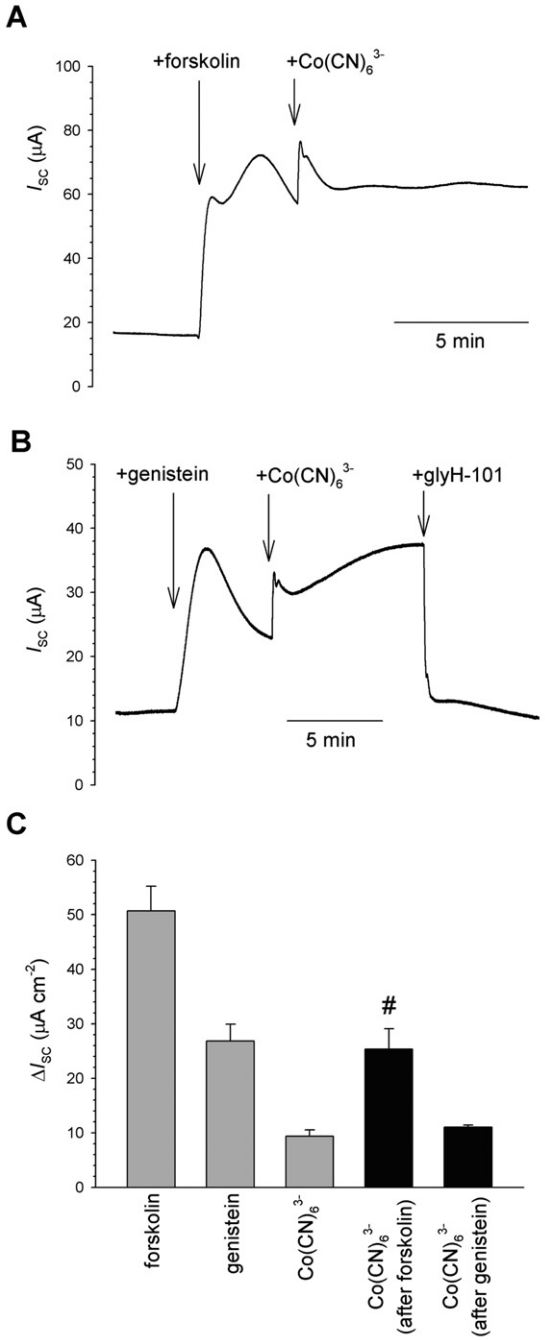

Figure 10

Additive effects of $Co(CN)_6^{3-}$ with forskolin and genistein on anion secretion. (A, B) Examples of the increase in I_{SC} in response to addition of maximally effective concentrations of forskolin (5 μM, A) or genistein (50 μM, B) to the apical face of a Calu-3 cell monolayer. Following stimulation with forskolin or genistein, addition of 10 mM $Co(CN)_6^{3-}$ to the apical solution stimulated a further increase in I_{SC}. (C) Columns indicate the mean change in I_{SC} following addition of forskolin (5 μM), genistein (50 μM) or $Co(CN)_6^{3-}$ (10 mM) to the apical face of previously unstimulated monolayers. Solid columns indicate the response to 10 mM $Co(CN)_6^{3-}$ following stimulation with either forskolin or genistein. # Indicates a significant difference from 10 mM $Co(CN)_6^{3-}$ alone ($P < 0.0005$). Mean of data from 3–9 monolayers.

however, this unusual property might make this anion a useful probe in future studies of Cl^- channel structure and function. Clearly, direct interference with Cl^- movement through the channel is not a desirable property of any putative CFTR potentiator.

While different pseudohalide anions showed different abilities to enter the channel pore, this does not seem to be related to their ability to increase CFTR conductance via interactions with cytoplasmic blocking substances. For example, the channel-blocking effects of pseudohalide anions were strongly voltage-dependent, with block usually being apparent only at depolarized voltages (Figure 8). This suggests that, as expected, these anions enter the pore more easily at depolarized voltages. Conversely, the apparent stimulating effects of these anions on macroscopic CFTR conductance in intact cells were stronger at more hyperpolarized voltages (Figures 2, 4). In other words, differences in voltage-dependence suggest that the effect of these anions on CFTR conductance in intact cells increases as their ability to enter the pore decreases, suggesting a non-pore-mediated effect of these anions on conductance. This suggestion of a non-pore-mediated effect is further strengthened by the effects of mutations that remove positive charges from the extracellular face of the CFTR protein. Thus the R334Q mutant, which neutralizes a pore lining positive charge that is known to attract extracellular anions into the outer mouth of the pore (Smith *et al.*, 2001; Gong and Linsdell, 2003c; Zhou *et al.*, 2007), did affect the ability of $Co(CN)_6^{3-}$ and $Co(NO_2)_6^{3-}$ to weaken apparent channel block in intact cells (Figures 5, 7). However, this apparent lack of pseudohalide sensitivity in R334Q appears to reflect weak cytoplasmic block under control conditions rather than a specific loss of external anion sensitivity of block (Figure 7). This interpretation is consistent with the R334Q mutation itself affecting cytoplasmic blocker interactions with CFTR (Zhou *et al.*, 2007). The effects of $Co(CN)_6^{3-}$ and $Co(NO_2)_6^{3-}$ were also sensitive to neutralization of positive charges on the fourth extracellular loop of CFTR (K892 and R899). Thus, the R899Q mutation showed similar sensitivity to current inhibition in intact cells as wild type, but this inhibition was completely insensitive to extracellular $Co(CN)_6^{3-}$ and $Co(NO_2)_6^{3-}$ (Figures 5, 7), suggesting a specific disruption of external anion effect in this mutant. Neutralization of another nearby positive charge, in K892Q, had a slightly different effect: block was slightly weakened relative to wild type, was unaffected by $Co(NO_2)_6^{3-}$ and was actually significantly strengthened in the presence of external $Co(CN)_6^{3-}$ (Figures 5, 7), again consistent with altered interactions between external anions and cytoplasmic blocking substances in this mutant. These two positively charged residues are not thought to be within the pore, as charge-changing mutations at these sites have no effect on permeation properties of the channel (Zhou *et al.*, 2008; Zhou and Linsdell, 2009). Based on our results with K892Q and R899Q (summarized in Figure 7), we speculate that pseudohalide anions interact with a site away from the channel pore to weaken channel interactions with cytoplasmic blocking substances and so promote elevated overall channel conductance. Neutralization of these positive charges also affected the ability of extracellular Cl^- to affect CFTR conductance via interaction with cytoplasmic blockers

(Figures 6, 7), implying that this effect of extracellular Cl^- ions might also be mediated by a non-pore-lining site on the CFTR protein. The effects of the K892Q and R899Q mutations point to some involvement of the fourth extracellular loop of CFTR in the regulation of CFTR by extracellular anions. We speculate that this large extracellular loop may either interact directly with extracellular anions, or be involved in transducing the effects of anions bound elsewhere. This suggestion indicates a need for more detailed investigation of the structure and function of this relatively poorly studied extracellular loop of the CFTR protein.

Clearly pseudohalide anions do not, themselves, represent a class of potential therapeutic compounds. Nevertheless, we believe their use demonstrates an important proof-of-principle: that regulation of CFTR channel conductance by extracellular substances can be targeted by non-physiological compounds acting on the CFTR protein itself. Currently known potentiators are thought to act at an intracellular site on the CFTR protein (Moran *et al.*, 2005; Zegarra-Moran *et al.*, 2007; Huang *et al.*, 2009). Our identification of an extracellular site for modulation of CFTR activity – possibly involving the fourth extracellular loop of the protein – therefore opens up a new opportunity for the development of CFTR potentiators acting by a novel molecular mechanism. Theoretically, such a mechanism of action, increasing Cl^- flux through open channels, should be additive with the effects of intracellularly active potentiators that act to increase channel open probability. Indeed, $Co(CN)_6^{3-}$ was still able to further increase anion secretion in Calu-3 cell monolayers following maximal stimulation by forskolin or genistein (Figure 10). In fact, the effects of $Co(CN)_6^{3-}$ appeared more than simply additive with those of forskolin, with this pseudohalide causing a greater increase in I_{SC} in forskolin-treated monolayers than in monolayers that had not received any prior stimulation (Figure 10). According to the mechanism we have proposed, extracellular pseudohalides should cause an increase in the conductance of all active CFTR channels. It is therefore likely that the magnitude of the response to $Co(CN)_6^{3-}$ will be somewhat proportional to the previous activity level of the CFTR channels, consistent with the larger effects of this substance following forskolin treatment to increase CFTR channel activity. Externally active CFTR potentiators that target the fourth extracellular loop of the protein might therefore provide a useful adjunct to other kinds of pharmacological treatments for CF, especially since combinations of drugs have already been suggested for the therapeutic treatment of CF (Verkman and Galietta, 2009; Anderson, 2010; Pedemonte *et al.*, 2011; Rogan *et al.*, 2011). Furthermore, since the proposed effects of pseudohalides are on the conductance of CFTR channels, we assume these effects will be preserved in most CF-mutant forms of CFTR that are trafficked to the cell membrane, with the possible exception of Class IV (defective conduction) mutants.

Acknowledgements

This work was supported by Research Grants from Cystic Fibrosis Canada to EAC and PL.

Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to receptors and channels (GRAC), 4th edition. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Alzamora R, King JD, Hallows KR (2011). CFTR regulation by phosphorylation. Methods Mol Biol 741: 471–488.

Anderson P (2010). Emerging therapies in cystic fibrosis. Ther Adv Respir Dis 4: 177–185.

Becq F, Mall MA, Sheppard DN, Conese M, Zegarra-Moran O (2011). Pharmacological treatment for cystic fibrosis: from bench to bedside. J Cyst Fibros 10 (Suppl. 2): S129–S145.

Cowley EA, Linsdell P (2002). Characterization of basolateral K^+ channels underlying anion secretion in the human airway cell line Calu-3. J Physiol 538: 747–757.

Cuthbert AW (2010). New horizons in the treatment of cystic fibrosis. Br J Pharmacol 163: 173–183.

Gadsby DC, Nairn AC (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. Physiol Rev 79: S77–S107.

Gadsby DC, Vergani P, Csanády L (2006). The ABC protein turned chloride channel whose failure causes cystic fibrosis. Nature 440: 477–483.

Ge N, Linsdell P (2006). Interactions between impermeant blocking ions in the CFTR chloride channel pore. Evidence for anion-induced conformational changes. J Membr Biol 210: 31–42.

Gong X, Linsdell P (2003a). Mutation-induced blocker permeability and multiion block of the CFTR chloride channel pore. J Gen Physiol 122: 673–687.

Gong X, Linsdell P (2003b). Coupled movement of permeant and blocking ions in the CFTR chloride channel pore. J Physiol 549: 375–385.

Gong X, Linsdell P (2003c). Molecular determinants and role of an anion binding site in the external mouth of the CFTR chloride channel pore. J Physiol 549: 387–397.

Gong X, Burbridge SM, Cowley EA, Linsdell P (2002). Molecular determinants of $Au(CN)_2^-$ binding and permeability within the cystic fibrosis transmembrane conductance regulator Cl^- channel pore. J Physiol 540: 39–47.

Guggino WB, Stanton BA (2006). New insights into cystic fibrosis: molecular switches that regulate CFTR. Nat Rev Mol Cell Biol 7: 426–436.

Hals GD, Stein PG, Palade PT (1989). Single channel characteristics of a high conductance anion channel in 'sarcoballs'. J Gen Physiol 93: 385–410.

Huang S-Y, Bolser D, Liu H-Y, Hwang T-C, Zou X (2009). Molecular modeling of the heterodimer of human CFTR's nucleotide-binding domains using a protein-protein docking approach. J Mol Graph Model 27: 822–828.

Illek B, Fischer H (1998). Flavonoids stimulate Cl^- conductance of human airway epithelium *in vitro* and *in vivo*. Am J Physiol 275: L902–L910.

Kidd JF, Kogan I, Bear CE (2004). Molecular basis for the chloride channel activity of cystic fibrosis transmembrane conductance regulator and the consequence of disease-causing mutations. Curr Top Dev Biol 60: 215–249.

Li C, Naren AP (2010). CFTR chloride channel in the apical compartments: spatiotemporal coupling to its interacting partners. Integr Biol 2: 161–177.

Li M-S, Holstead RG, Wang W, Linsdell P (2011). Regulation of CFTR chloride channel macroscopic conductance by extracellular bicarbonate. Am J Physiol 300: C65–C74.

Linsdell P, Hanrahan JW (1998). Adenosine triphosphate-dependent asymmetry of anion permeation in the cystic fibrosis transmembrane conductance regulator chloride channel. J Gen Physiol 111: 601–614.

Linsdell P, Tabcharani JA, Hanrahan JW (1997). Multi-ion mechanism for ion permeation and block in the cystic fibrosis transmembrane conductance regulator chloride channel. J Gen Physiol 110: 365–377.

Moran O, Galietta LJV, Zegarra-Moran O (2005). Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. Cell Mol Life Sci 62: 446–460.

Moser SL, Harron SA, Crack J, Fawcett JP, Cowley EA (2008). Multiple KCNQ potassium channel subtypes mediate basal anion secretion from the human airway epithelial cell line Calu-3. J Membr Biol 221: 153–163.

Pedemonte N, Tomati V, Sondo E, Caci E, Millo E, Armirotti A *et al.* (2011). Dual activity of aminoarylthiazoles on the trafficking and gating defects of the cystic fibrosis transmembrane conductance regulator chloride channel caused by cystic fibrosis mutations. J Biol Chem 286: 15215–15226.

Rogan MP, Stoltz DA, Hornick DB (2011). Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. Chest 139: 1480–1490.

Shen B-Q, Finkbeiner WE, Wine JJ, Mrsny RJ, Widdicombe JH (1994). Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl^- secretion. Am J Physiol 266: L493–L501.

Singh M, Krouse ME, Moon S, Wine JJ (1997). Most basal I_{sc} in Calu-3 human airway cells is bicarbonate-dependent Cl^- secretion. Am J Physiol 272: L690–L698.

Smith SS, Liu X, Zhang Z-R, Sun F, Kriewall TE, McCarty NA *et al.* (2001). CFTR: covalent and noncovalent modification suggests a role for fixed charges in anion conduction. J Gen Physiol 118: 407–431.

Verkman AS, Galietta LJV (2009). Chloride channels as drug targets. Nat Rev Drug Discov 8: 153–171.

Wright AM, Gong X, Verdon B, Linsdell P, Mehta A, Riordan JR *et al.* (2004). Novel regulation of cystic fibrosis transmembrane conductance regulator (CFTR) channel gating by external chloride. J Biol Chem 279: 41658–41663.

Zegarra-Moran O, Monteverde M, Galietta LJV, Moran O (2007). Functional analysis of mutations in the putative binding site for cystic fibrosis transmembrane conductance regulator potentiators. Interaction between activation and inhibition. J Biol Chem 282: 9098–9104.

Zhou J-J, Linsdell P (2009). Evidence that extracellular anions interact with a site outside the CFTR chloride channel pore to modify channel properties. Can J Physiol Pharmacol 87: 387–395.

Zhou J-J, Fatehi M, Linsdell P (2007). Direct and indirect effects of mutations at the outer mouth of the cystic fibrosis transmembrane conductance regulator chloride channel pore. J Membr Biol 216: 129–142.

Zhou J-J, Fatehi M, Linsdell P (2008). Identification of positive charges situated at the outer mouth of the CFTR chloride channel pore. Pflugers Arch 457: 351–360.

Zhou J-J, Li M-S, Qi J, Linsdell P (2010). Regulation of conductance by the number of fixed positive charges in the intracellular vestibule of the CFTR chloride channel pore. J Gen Physiol 135: 229–245.

Zhou Z, Hu S, Hwang T-C (2001). Voltage-dependent flickery block of an open cystic fibrosis transmembrane conductance regulator (CFTR) channel pore. J Physiol 532: 435–448.