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RESEARCH PAPER

Cysteine-independent inhibition of the CFTR chloride channel by the cysteine-reactive reagent sodium (2-sulphonatoethyl) methanethiosulphonate

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Background and purpose: Methanethiosulphonate (MTS) reagents are used extensively to modify covalently cysteine side chains in ion channel structure-function studies. We have investigated the interaction between a widely used negatively charged MTS reagent, (2-sulphonatoethyl) methanethiosulphonate (MTSES), and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel.
Experimental approach: Patch clamp recordings were used to study a 'cys-less' variant of human CFTR, in which all 18 endogenous cysteine residues have been removed by mutagenesis, expressed in mammalian cell lines. Use of excised inside–out membrane patches allowed MTS reagents to be applied to the cytoplasmic face of active channels.
Key results: Intracellular application of MTSES, but not the positively charged MTSET, inhibited the function of cys-less CFTR. Inhibition was voltage dependent, with a K_d of 1.97 mmol·L^{-1} at –80 mV increasing to 36 mmol·L^{-1} at +80 mV. Inhibition was completely reversed on washout of MTSES, inconsistent with covalent modification of the channel protein. At the single channel level, MTSES caused a concentration-dependent reduction in unitary current amplitude. This inhibition was strengthened when extracellular Cl^- concentration was decreased.
Conclusions and implications: Our results indicate that MTSES inhibits the function of CFTR in a manner that is independent of its ability to modify cysteine residues covalently. Instead, we suggest that MTSES functions as an open channel blocker that enters the CFTR channel pore from its cytoplasmic end to physically occlude Cl^- permeation. Given the very widespread use of MTS reagents in functional studies, our findings offer a broadly applicable caveat to the interpretation of results obtained from such studies.
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Keywords: chloride channel; cysteine reactive reagents; cystic fibrosis transmembrane conductance regulator; methanethiosulphonate; MTSES; MTSET; open channel block; patch clamp; substituted cysteine accessibility mutagenesis

Abbreviations: BHK, baby hamster kidney; CFTR, cystic fibrosis transmembrane conductance regulator; MTS, methanethiosulphonate; MTSEA, 2-aminoethyl methanethiosulphonate; MTSES, (2-sulphonatoethyl) methanethiosulphonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulphonate; PKA, protein kinase A catalytic subunit; PPi, pyrophosphate; SCAM, substituted cysteine accessibility mutagenesis

Introduction

Substituted cysteine accessibility mutagenesis (SCAM) is an extremely widespread and powerful technique for studying the structure and function of membrane proteins such as ion channels (Karlin and Akabas, 1998; Zhu and Casey, 2007). The principle behind this technique is that the covalent reaction between small hydrophobic substances [usually methanethiosulphonate (MTS) reagents] and solvent-accessible cysteine side chains will lead to irreversible labelling of the protein and, in most cases, a measurable change in function. Most useful in this regard have been the MTS reagents bearing different charges, such as the positively charged [2-(trimethylammonium)ethyl] methanethiosulphonate (MTSET) and the negatively charged (2-sulphonatoethyl) methanethiosulphonate (MTSES). The utility of these substances comes from their rapid and specific reaction with cysteine sulphydryl groups at the water-accessible surface of the protein. In addition to the identification of pore-forming regions in ion channel proteins, SCAM has been used to identify different conformational states, to locate channel gates and ion selectivity filters and to generate information on

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the electrostatic potential profile within channel pores (Karlin and Akabas, 1998). It is difficult to identify a class of ion channel proteins for which important structure-function information has not been obtained using some form of SCAM approach. For example, at least seven different research groups have published results concerning the interaction between MTSES and/or MTSET and mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR) (Cheung and Akabas, 1996; Fu and Kirk, 2001; Smith *et al.*, 2001; Zhang *et al.*, 2005; Cui *et al.*, 2006; St. Aubin and Linsdell 2006; Beck *et al.*, 2008), an epithelial Cl^- channel that is mutated in the fatal genetic disease cystic fibrosis (Gadsby *et al.*, 2006).

In many cases, the potential impact of SCAM-based techniques is considered so great that researchers are willing to generate 'cys-less' forms of their protein of interest in which all endogenous cysteine residues have been removed by mutagenesis. Again, at least three different groups have independently generated cys-less versions of human CFTR by mutating all 18 endogenous cysteine residues to serine or leucine residues (Cui *et al.*, 2006; Mense *et al.*, 2006; Wang *et al.*, 2007). Using patch clamp recording, we now show that MTSES, but not MTSET, inhibits the function of cys-less CFTR when applied to the cytoplasmic face of excised membrane patches. This effect of MTSES is shown to be inconsistent with covalent modification of cysteine residues. Instead, we suggest that MTSES inhibition reflects direct block of the open channel pore by this negatively charged molecule. These results raise important caveats concerning the use of MTSES to probe the structure and function of anion-selective channel pores.

Methods

Expression of cys-less CFTR in mammalian cells

The cys-less CFTR used in the present manuscript was that described by Mense *et al.* (2006) and was a kind gift of Dr David Gadsby (Rockefeller University, New York, USA). The full coding sequence was subcloned into the pIRES2-EGFP vector using *SacI* and *XmaI* restriction enzyme sites. To facilitate channel protein maturation and expression in the cell membrane, the V510A mutation (Wang *et al.*, 2007) was introduced into this construct using the QuikChange site directed mutagenesis system as described previously (Gong *et al.*, 2002) and verified by DNA sequencing. Baby hamster kidney (BHK) and human embryonic kidney cells were transiently transfected with pIRES2-EGFP-cys-less/V510A CFTR cDNA as described previously (Gong *et al.*, 2002), except that 24 h after transfection, cells were transferred to 27°C to promote mature protein expression (see Supporting Information, Figure S1). Cells were used for electrophysiological experimentation after 1–3 days at 27°C.

Electrophysiological recordings

Macroscopic and single channel patch clamp recordings were made from inside–out membrane patches excised from BHK cells as described in detail previously (Gong *et al.*, 2002; Ge *et al.*, 2004; St. Aubin and Linsdell 2006). Once trafficked to the cell membrane (see Supporting Information, Figure S1), the properties of cys-less/V510A CFTR channel currents appeared identical to those of wild-type CFTR studied previously, with the exception that single channel conductance appeared increased (see Figures 2 and 3). Following patch excision and recording of background currents, CFTR channels were activated by exposure to protein kinase A catalytic subunit (PKA; 1–20 $nmol{\cdot}L^{-1}$) plus adenosine 5′-triphosphate magnesium salt (MgATP; 1 $mmol{\cdot}L^{-1}$) in the cytoplasmic solution. Macroscopic currents were recorded after treatment with sodium pyrophosphate (PPi; 2 $mmol{\cdot}L^{-1}$) to maximize channel open probability and ensure congruence of macroscopic and single channel current–voltage relationships (Linsdell and Gong, 2002; Gong and Linsdell, 2003; Fatehi *et al.*, 2007). The intracellular (bath) solution contained ($mmol{\cdot}L^{-1}$): NaCl 150, $MgCl_2$ 2, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonate 10. The extracellular (pipette) solution was either the same as the bath solution (high [Cl^-]) or following replacement of NaCl with 150 $mmol{\cdot}L^{-1}$ Na gluconate [low (Cl^-)]. All experimental solutions were adjusted to pH 7.4 using NaOH. Membrane currents were filtered at 50 Hz (for single channel currents) or 100 Hz (for macroscopic currents) using an 8-pole Bessel filter, digitized at 250 Hz (single channel currents) or 1 kHz (macroscopic currents), and recorded using pCLAMP9 software (Molecular Devices, Sunnyvale, CA, USA). Macroscopic current–voltage relationships were constructed using depolarizing voltage ramp protocols (Linsdell and Hanrahan, 1996a; 1998). Background (leak) currents recorded before addition of PKA and adenosine 5′-triphosphate (ATP) have been subtracted digitally, leaving uncontaminated CFTR currents (Linsdell and Hanrahan, 1998; Gong and Linsdell, 2003). Recordings were made at room temperature, 21–24°C.

Treatment with MTS reagents

Both MTSES and MTSET were initially prepared as 160 $mmol{\cdot}L^{-1}$ stock solutions in distilled water and stored frozen at –20°C as small volume aliquots until the time of use, when they were diluted in bath solution and used immediately. Following recording of control channel activity, MTS reagents were added directly to the bath at final concentrations of 0.2–6 $mmol{\cdot}L^{-1}$.

Data analysis and statistical procedures

The effects of MTS reagents on current amplitudes were expressed as the fractional current, that is, the current in the presence of drug as a fraction of the control current recorded before addition of the drug. Concentration–inhibition relationships (Figure 1C) were fitted by the equation:

$$\text{Fractional current} = 1/\left(1 + \{[\text{MTSES}]/K_d\}^{n\text{H}}\right) \quad (1)$$

where K_d is the dissociation constant and n_H is the slope factor or Hill coefficient.

The voltage-dependence of K_d was quantified using the Woodhull (1973) model of voltage-dependent block, in which the K_d at voltage V is given by:

$$K_d(\text{V}) = K_d(0)\exp(-z\delta \text{V}F/RT) \quad (2)$$

where $z\delta$ is the effective valence of the blocking ion (actual valence multiplied by the fraction of the transmembrane elec-

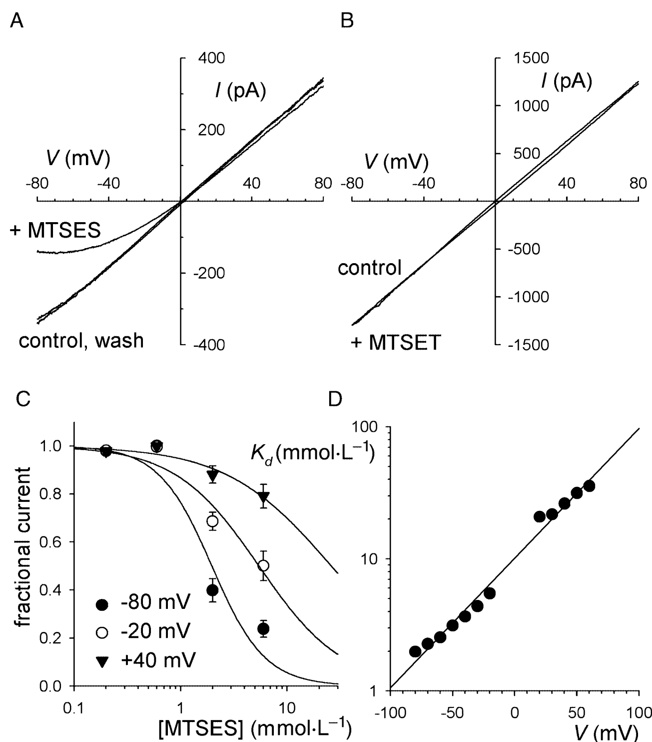

Figure 1 Block of cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ current by intracellular (2-sulphonatoethyl) methanethiosulphonate (MTSES). (A) An example of the leak-subtracted macroscopic current–voltage relationship for cys-less/V510A-CFTR following maximal current stimulation with protein kinase A catalytic subunit (PKA), adenosine 5′-triphosphate (ATP) and pyrophosphate (PPi). Current from the same patch is shown before (control) and immediately following addition of 2 mmol·L^{-1} MTSES (+MTSES) to the intracellular solution, and also following washing of MTSES from the bath and re-application of PKA, ATP and PPi (wash). (B) A similar example of the current–voltage relationship recorded before (control) and immediately following addition of 2 mmol·L^{-1} [2-(trimethylammonium)ethyl] methanethiosulphonate (+MTSET) to the intracellular solution. (C) Mean fractional current remaining following the addition of different concentrations of MTSES, measured at membrane potentials of –80 mV, –20 mV and +40 mV. Mean of data from 4–5 patches. Each set of data has been fitted by Equation (1) as described in Methods, with the following parameters: at –80 mV, K_d = 1.97 mmol·L^{-1}, n_H = 1.57; at –20 mV, K_d = 5.47 mmol·L^{-1}, n_H = 1.11; and at +40 mV, K_d = 26.1 mmol·L^{-1}, n_H = 0.88. (D) Relationship between mean K_d [estimated using fits such as those shown in (C)] and membrane potential. The data have been fitted by Equation (2), with $K_d(0)$ = 11.2 mmol·L^{-1} and $z\delta$ = –0.52.

tric field apparently experienced during the blocking reaction) and *F*, *R* and *T* have their usual thermodynamic meanings.

Effects of single concentrations of MTSES on unitary current amplitudes were quantified by fitting the fractional current–voltage relationship (Figure 3C,D) with the equation:

$$\text{Fractional current} = K_d(\text{V})/(K_d(\text{V}) + [\text{MTSES}]) \qquad (3)$$

Values are presented as mean ± standard error of the mean (SEM) of data from *n* membrane patches. For graphical presentation, error bars represent ± SEM and, where no error bars are shown, this is smaller than the size of the symbol. Tests of significance were carried out using Student's two-tailed *t*-test; a *P* value of <0.05 was considered to be statistically significant.

Nomenclature

The nomenclature in this paper conforms to the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al.*, 2008).

Chemical reagents and materials

PKA was obtained from Promega (Madison, WI, USA). MTS reagents were from Toronto Research Chemicals (North York, ON, Canada). All other chemicals were from Sigma-Aldrich (Oakville, ON, Canada). pIRES2-EGFP vector was from Clontech (Mountain View, CA, USA), and QuikChange site-directed mutagenesis system, Stratagene (La Jolla, CA, USA).

Results

Expression of cys-less/V510A CFTR in BHK cells led to the appearance of PKA- and ATP-dependent PPi-stimulated macroscopic currents when the cells were grown at 27°C. Examples of such currents, which had similar properties to those of wild-type CFTR studied many times previously (e.g. Linsdell and Hanrahan, 1998; Gong *et al.*, 2002), are shown in Figure 1. Application of the negatively charged cysteine reactive reagent MTSES to the intracellular solution led to voltage-dependent inhibition of these currents, with strong inhibition at hyperpolarized voltages but only minor effects at depolarized voltages (Figure 1A). This inhibition does not appear to reflect covalent modification of CFTR or associated proteins, since the effects of MTSES could be completely reversed by washing from the bath followed by reapplication of PKA, ATP and PPi (Figure 1A). In contrast to these effects of MTSES, the positively charged MTSET was without significant effect on current amplitudes (Figure 1B). The concentration- and voltage-dependent effects of MTSES under these conditions are shown in Figure 1C,D, indicating a K_d in the millimolar range at hyperpolarized voltages (Figure 1D). Fitting the K_d–voltage relationship with Equation (2) suggested a K_d of 11.2 mmol·L^{-1} at 0 mV and a $z\delta$ of –0.52 (Figure 1D).

At the single channel level, cytoplasmic application of MTSES to inside–out membrane patches led to a clear reduction in CFTR unitary current amplitude that was most pronounced at hyperpolarized voltages. This apparent mechanism of inhibition was observed both with a high extracellular Cl$^-$ concentration (154 mmol·L^{-1}; Figure 2) and also when extracellular Cl$^-$ concentration was reduced to 4 mmol·L^{-1} (Figure 3). Importantly, unitary current amplitude appeared stable in the presence of MTSES, and channel openings to full (control) current amplitude were not observed under these conditions. As with effects on macroscopic currents, these effects of MTSES on unitary current amplitude were fully reversed by washing MTSES from the intracellular solution (not shown). The degree and voltage dependence of inhibition of unitary current amplitude observed following application of 2 mmol·L^{-1} MTSES (Figure 3C) or 6 mmol·L^{-1} MTSES (Figure 3D) was similar to that observed for macroscopic currents under the same ionic conditions (Figure 1), suggesting that this reduction in unitary current amplitude is the major mechanism by which MTSES inhibits CFTR Cl$^-$

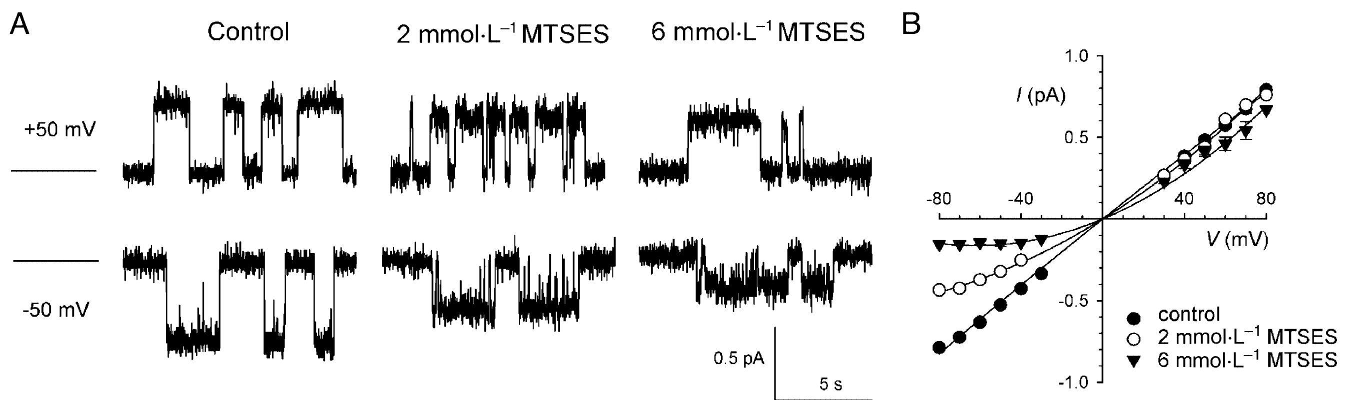

Figure 2 Effect of (2-sulphonatoethyl) methanethiosulphonate (MTSES) on cystic fibrosis transmembrane conductance regulator (CFTR) single channel currents. (A) An example of the single channel currents recorded at membrane potentials of +50 mV and −50 mV as indicated. In each case, the closed state of the channel is indicated by the line on the far left. Currents were recorded in the absence of MTSES (control) or with 2 or 6 mmol·L^{-1} MTSES present in the intracellular solution as indicated. (B) Mean unitary current–voltage relationships recorded in the absence of MTSES (control) and with 2 mmol·L^{-1} or 6 mmol·L^{-1} MTSES present in the intracellular solution. Mean of data from 3–8 patches. Mean slope conductance measured under control conditions was 10.0 ± 0.1 pS (n = 10), somewhat larger than the value we measured for wild-type CFTR under identical conditions (~8.5 pS; Fatehi *et al.*, 2007; Zhou *et al.*, 2007). A small increase in unitary conductance has previously been reported in cys-less CFTR (Mense *et al.*, 2006).

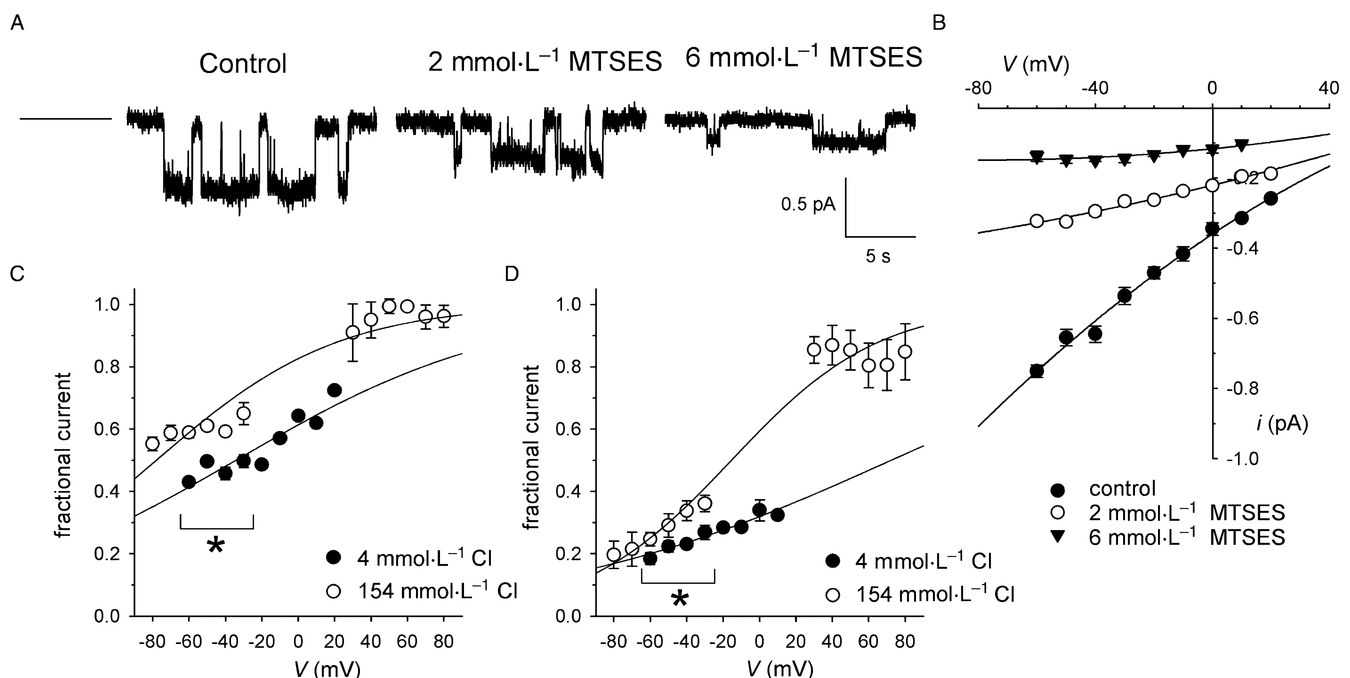

Figure 3 Inhibition of single channel current amplitude by (2-sulphonatoethyl) methanethiosulphonate (MTSES) is dependent on extracellular Cl$^-$ concentration. (A) An example of single channel currents recorded at a membrane potential of −30 mV with a low extracellular Cl$^-$ concentration (4 mmol·L^{-1}). The closed state of the channel is indicated by the line on the far left. Currents were recorded in the absence of MTSES (control) or with 2 or 6 mmol·L^{-1} MTSES present in the intracellular solution as indicated. (B) Mean unitary current–voltage relationships recorded under these conditions in the absence of MTSES (control) and with 2 mmol·L^{-1} or 6 mmol·L^{-1} MTSES present in the intracellular solution. Mean of data from 3–6 patches. Mean slope conductance measured under control conditions was 6.7 ± 0.5 pS (n = 5), again somewhat larger than the value we measured for wild-type cystic fibrosis transmembrane conductance regulator under identical conditions (5.4–5.7 pS; Ge and Linsdell (2006); St. Aubin and Linsdell 2006). (C,D) Mean reduction in unitary current amplitude in response to addition of 2 mmol·L^{-1} (C) or 6 mmol·L^{-1} MTSES (D), under conditions of high external (Cl$^-$) (154 mmol·L^{-1}; see Figure 2) or low external (Cl$^-$) (4 mmol·L^{-1}). Where the voltage ranges studied under different ionic conditions overlap, the fraction of control current remaining was significantly less with 4 mmol·L^{-1} extracellular Cl$^-$ than with 154 mmol·L^{-1} Cl$^-$ (P < 0.05), both at 2 mmol·L^{-1} and 6 mmol·L^{-1} MTSES, as indicated by asterisks. These mean data have been fitted by Equation (3) with the following parameters: (C) for 154 mmol·L$^{-1'}$ Cl$^-$: $K_d(0)$ = 9.5 mmol·L^{-1}, $z\delta$ = −0.51, for 4 mmol·L^{-1} Cl$^-$: $K_d(0)$ = 3.2 mmol·L^{-1}, $z\delta$ = −0.34; (D) for 154 mmol·L^{-1} Cl$^-$: $K_d(0)$ = 8.8 mmol·L^{-1}, $z\delta$ = −0.63, for 4 mmol·L^{-1} Cl$^-$: $K_d(0)$ = 2.8 mmol·L^{-1}, $z\delta$ = −0.27.

currents. Interestingly, the degree of inhibition appears to be greater at low extracellular Cl^- concentrations (Figure 3C,D), and indeed, where the voltage ranges studied under different ionic conditions overlap (–60 to –30 mV), the fraction of control current remaining was significantly smaller with 4 $mmol \cdot L^{-1}$ extracellular Cl^- than with 154 $mmol \cdot L^{-1}$ Cl^- ($P <$ 0.05), both at 2 $mmol \cdot L^{-1}$ and 6 $mmol \cdot L^{-1}$ MTSES. Fitting of the fractional current–voltage relationship data from individual patches with Equation (3) suggested a $K_d(0)$ of 9.7 ± 1.5 $mmol \cdot L^{-1}$ (n = 6) with 154 $mmol \cdot L^{-1}$ Cl^-, which was significantly reduced to 3.1 ± 0.1 $mmol \cdot L^{-1}$ (n = 6) ($P < 0.001$) with 4 $mmol \cdot L^{-1}$ Cl^-, and a $z\delta$ of –0.55 ± 0.05 (n = 6) with 154 $mmol \cdot L^{-1}$ Cl^-, significantly reduced to –0.31 ± 0.03 (n = 6) ($P < 0.005$) with 4 $mmol \cdot L^{-1}$ Cl^-.

Discussion and conclusions

The inhibitory effects of intracellular MTSES on cys-less CFTR are inconsistent with covalent modification of cysteines or other residues. The CFTR construct used in the present study has had all 18 endogenous cysteines removed (Mense *et al.*, 2006). Furthermore, the inhibitory effects of MTSES could be fully reversed by washing (Figure 1A), suggesting that the drug was never covalently attached to its target. The concentration-dependent effect of MTSES on unitary current amplitude (Figures 2 and 3) suggests a concentration-dependent occupancy of a binding site by the drug, in contrast to the 'all-or-none' functional effect expected for a covalent modification event (e.g. Zhang *et al.*, 2005).

We suggest that intracellular MTSES is acting as an open channel blocker that physically occludes Cl^- permeation through the CFTR channel pore, resulting in an overall decrease in the rate of Cl^- movement. The effects of MTSES – including its voltage dependence, concentration- dependence and dependence on extracellular Cl^- concentration – are those expected of an open channel blocker (Hwang and Sheppard, 1999; Cai *et al.*, 2004; Linsdell, 2006), and indeed are reminiscent of a number of well-known CFTR channel blockers for which this mechanism of action has been demonstrated (Linsdell, 2005). The effect of MTSES on unitary current amplitude suggests that its open channel blocking action is kinetically 'fast', with individual blocking and unblocking events being too brief to be resolved by patch clamp recording. This same mechanism has previously been demonstrated for intracellular CFTR channel blockers such as gluconate (Linsdell and Hanrahan, 1996b), tolbutamide (Venglarik *et al.*, 1996), taurolithocholate-3-sulphate (Linsdell and Hanrahan, 1999), butyrate (Linsdell, 2001) and niflumic acid (Scott-Ward *et al.*, 2004). Indeed, it has been proposed that the CFTR channel pore has a wide inner vestibule that allows the relatively non-specific binding of a broad range of large cytoplasmic anions that results in channel block (Linsdell, 2006); our results suggest that MTSES can be added to this list of blocking anions. Small intracellular anionic blockers with this mechanism of action have previously been shown to interact strongly with a positively charged amino acid side chain in the intracellular vestibule of the pore that is contributed by lysine residue K95 (Linsdell, 2005). We hypothesize that MTSES may also enter into the pore and interact with K95, although we have not tested this hypothesis directly.

Often, these same large blocking anions do not inhibit CFTR currents when added to the extracellular solution, and as a result, block is 'asymmetric'; this has been proposed to reflect asymmetric structure of the channel pore, with the outer mouth of the pore being more physically restricted than the wide inner vestibule (Hwang and Sheppard, 1999; Linsdell, 2006). Consistent with this proposed asymmetry, it has been reported by several different groups that extracellular MTSES, even at millimolar concentrations, has no effect on currents carried by wild-type CFTR (Cheung and Akabas, 1996; Smith *et al.*, 2001; Beck *et al.*, 2008; Fatehi and Linsdell, 2008).

Our results provide important caveats for the use of MTS reagents such as MTSES to modify ion channel function. At high concentrations, this cysteine reactive reagent has direct effects on Cl^- permeation in the CFTR pore. This could be misinterpreted as being the result of charge deposition in the permeation pathway due to covalent modification of a cysteine side chain. For example, we previously showed that intracellular application of a low concentration of MTSES (200 $\mu mol \cdot L^{-1}$) led to outward rectification of the macroscopic current–voltage relationship in a CFTR mutant (R303C) but not wild-type CFTR, which we proposed was due to the electrostatic effects of the addition of a negative charge close to the inner mouth of the pore by covalent modification of the introduced cysteine side chain (St. Aubin and Linsdell 2006). The change in macroscopic current–voltage relationship shape observed in MTSES-modified R303C-CFTR is, however, similar to that resulting from voltage-dependent block by MTSES (Figure 1A). Nevertheless, it seems unlikely that open channel block contributed to the reported effects of MTSES on R303C-CFTR, since (i) qualitatively opposite effects were observed with positively charged MTSET, and (ii) these effects were observed at MTSES concentrations of only 200 $\mu mol \cdot L^{-1}$, a concentration which does not result in significant current blockage (Figure 1).

In our previous study, we also showed that intracellular MTSES and MTSET caused rapid rundown of wild-type CFTR currents (St. Aubin and Linsdell 2006). The absence of such a run-down effect in the present study on cys-less CFTR suggests that this effect was due to modification of endogenous, intracellularly accessible cysteines.

Application of MTSES to the extracellular solution has previously been shown to inhibit many different cysteine mutant forms of CFTR (Akabas *et al.*, 1994; Cheung and Akabas, 1996; Akabas, 1998; Liu *et al.*, 2004; Beck *et al.*, 2008), in some cases in a voltage-dependent manner (Smith *et al.*, 2001), although in no case has an effect on wild-type CFTR been reported. While in the abovementioned cases inhibition does appear to reflect the effects of covalent modification of the introduced cysteine side chain rather than any kind of channel blocking effect, our current findings stress the importance of appropriate control experiments for these kinds of studies, such as demonstration of the irreversible nature of the inhibition on MTSES washout, reversal by application of reducing agents, lack of effect of MTSES following mutagenesis to residues other than cysteine, and demonstration of functional effects of other MTS reagents such as positively charged MTSET or 2-aminoethyl methanethiosulphonate (MTSEA).

Another caveat raised by the present study is that cysteine mutations in the CFTR channel pore could alter either the

affinity of MTSES open channel block or the accessibility of MTSES to its binding site within the pore, and this could be misinterpreted as modification of the introduced cysteine side chain. For example, mutations within the pore that strengthen the blocking effects of MTSES could, in theory, lead to an increase in apparent MTSES sensitivity without promoting modification of cysteine side chains. While mutagenesis of positively charged amino acids such as K95 (Linsdell, 2005), R303 (St. Aubin *et al*., 2007; Zhou *et al*., 2007) and R334 (Zhou *et al*., 2007) have been shown, directly or indirectly, to decrease sensitivity to open channel blockers, we are unaware of mutations that lead to a dramatic increase in blocker sensitivity.

Mutations in the pore could also, in theory, allow access of extracellular MTSES to its putative blocking site in the pore inner vestibule, leading indirectly to an increase in apparent sensitivity to extracellular MTSES. Mutations within the narrow region of the pore in particular have previously been shown to affect the apparent affinity of blocker binding by changing blocker movement within the pore rather than binding per se (Gong and Linsdell, 2003; Fatehi *et al*., 2007). Modification of cysteines substituted for narrow pore region residues F337, T338 and S341 by extracellular MTS reagents has been reported previously (Cheung and Akabas, 1996; Liu *et al*., 2004; Fatehi and Linsdell, 2008), although these cysteine mutants were sensitive not only to MTSES but also to positively charged MTSET or MTSEA.

Since the blocker sensitivity of different types of Cl^- channels is highly overlapping (Jentsch *et al*., 2002; Hartzell *et al*., 2005), the caveats raised above might relate not only to studies of CFTR but to MTS modification studies of all Cl^- channel pores in general. Given the widespread use of MTS reagents in the functional characterization of ion channels, these cysteine-independent effects of MTSES are of broad significance in the interpretation of the effects of such compounds. Unless shown otherwise, it would be prudent to consider MTSES as a substance that is capable of entering into anion-selective channel pores and altering their functional properties in a cysteine-independent manner.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative example of a Western blot for cystic fibrosis transmembrane conductance regulator (CFTR) using protein from human embryonic kidney (HEK293) cells transiently transfected with wild-type CFTR, cys-less CFTR or cys-less/V510A CFTR, and grown in culture at the temperature indicated.

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