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A possible role for intracellular GSH in spontaneous reaction of a cysteine (T338C) engineered into the Cystic Fibrosis Transmembrane Conductance Regulator

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Abstract The conductance of oocytes expressing T338C CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) exhibits variable responses to dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) that we proposed might be due to the extraction of copper from an adventitious binding site (Liu et al. J Biol Chem 281(12):8275–8285, 2006). In order to study the origins of variability in chemical reactivity of T338C CFTR channels, oocytes expressing T338C CFTR were exposed to BCNU (bischloroethylnitrosourea), an inhibitor of glutathione reductase. BCNU treatment caused a significant reduction of initial conductance and an increase in the response to 2-ME or DTT, suggesting a direct or indirect influence of intracellular glutathione (GSH), a major determinant of the disposition of intracellular copper. Single-channel recordings indicated that T338C CFTR channels not exposed to 2-ME or DTT exhibited multiple conductance levels not seen in T338A CFTR channels. Exposure to BCNU shifted the distribution of single-channel current amplitudes towards lower values, whereas exposure to DTT favored higher amplitudes. These results suggest that the altered chemical state of T338C channels is associated with a decreased single-channel conductance and that intracellular factors (most likely GSH) may modulate the propensity of the channel to form these altered states.

Keywords CFTR · Copper · Glutathione · Engineered cysteine · Channel

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Introduction

We previously reported that a cysteine substituted into transmembrane segment 6 (TM6) of CFTR can undergo spontaneous changes in its chemical state. We proposed that the cysteine substituted at 338 was a component of an adventitious metal binding site exhibiting a high affinity for copper (Liu et al. 2006). However, the origin of the variability in the chemical reactivity among oocytes expressing T338C CFTR was not fully understood, nor was the basis for the changes in macroscopic conductance discerned, i.e., change in single-channel conductance or gating (open probability). Because intracellular GSH has been demonstrated to be an important determinant of the disposition of intracellular copper (Freedman et al. 1989; Ciriolo et al. 1990; Ascone et al. 1993; Ferreira et al. 1993), I used two-electrode-voltage-clamp (TEVC) and single-channel recording to examine the effects of changes in intracellular GSH on the spontaneous reactions of T338C CFTR. BCNU inhibits glutathione reductase (Shinohara and Tanaka 1979; Eklow et al. 1984) and has been shown to

produce a decrease in GSH concentration in rat hepatocytes (Eklow et al. 1984; Meredith and Reed 1983) and rabbit renal proximal tubules (Messana et al. 1988). Exposure to BCNU decreased the initial conductance of oocytes expressing T338C CFTR and increased the magnitude of the response to 2-ME or DTT, as if a lower level of cellular GSH promoted the modified state of the cysteine. These results are consistent with the idea that intracellular GSH might be responsible, at least in part, for the variability in the chemical state of T338C CFTR.

Materials and methods

Mutagenesis and in vitro transcription

The methods used for mutagenesis and in vitro transcription were similar to those reported previously (Liu et al. 2006; Smith et al. 2001). Briefly, CFTR mutants were generated using the QuickChange™ site-directed mutagenesis kit from Stratagene. The sequences in the region of the mutation and in the whole PCR generated region were confirmed by direct DNA sequencing. The CFTR cRNAs for *Xenopus* oocyte injection were synthesized using the in vitro transcription kit, mMessage mMachine (Ambion, Inc., Austin, TX). The transcription products were purified and the quality and quantity of the transcripts were assessed on an agarose gel.

Oocyte preparation and whole-cell electrophysiological recordings

Protocols for preparing oocytes and the methods for whole-cell recordings were identical to those described in Liu et al. (2004). Briefly, oocytes were defoliculated using enzymatic digestion, then stored at 18°C in modified Barth's Solution (MBSH) containing in mM: 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2 4H_2O$, 0.41 $CaCl_2 2H_2O$, 2.4 $NaHCO_3$, 10 HEPES-HemiNa and 250mg/l Amikacin. Oocytes were then injected with 50 nl CFTR cRNA at ~0.2 ng/nl with the cRNA encoding human β_2-adrenergic receptor. Higher concentrations of cRNAs were injected for single-channel recordings (~2–10 ng/nl).

For whole-cell recordings, individual oocytes were placed in the recording chamber and continuously perfused at room temperature with Frog Ringer solution containing in mM: 98 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES-HemiNa, pH 7.4. Whole-cell data were acquired using a two-electrode voltage clamp amplifier (TEVC-200, Dagan Corporation) and data acquisition software (pClamp 8, Axon Instruments Inc). Oocytes were maintained under open circuit conditions and the membrane potential was periodically ramped from –120 mV to +60 mV over a span of 1.8 s to construct the whole-cell I–V plots. The CFTR conductance was monitored as the conductance resulting from exposure to stimulatory cocktail containing 10 μM isoproterenol (Isop) and 1 mM isobutylmethyl xanthine (IBMX), a phosphodiesterase inhibitor. An analysis program developed in the Dawson lab was used to analyze data. The conductance was calculated from the slope of the I–V plot at the reversal potential (g_{Cl} at $V_m = E_{rev}$) using a voltage range from $V_m = E_{rev} - 10$ mV to $V_m = E_{rev} + 10$ mV. Data are reported as Mean ± SEM.

Single-channel recordings

The methods for single-channel recordings were similar to that described in Liu et al. (2004). Briefly, single CFTR channels were studied at room temperature in excised, inside-out patches formed from oocytes after the vitelline membrane had been manually removed. Pipettes were pulled in 4–6 stages from borosilicate glass (Sutter Instruments) and had resistances averaging ~5–10 MΩ when filled with pipette solution. The pipette solution contained (in mM): 142 NMDG-Cl, 1.8 CaCl2, 2 $MgCl_2$, 5 HEPES HemiNa, pH 7.4. 5 MES was used in place of HEPES when a pH of 6 was desired for the pipette solution. Typical seal resistances ranged from 50 to 200 GΩ. Channels were activated by PKA (Promega) following excision into an intracellular solution (146 NMDG-Cl, 2 $MgCl_2$, 0.5 Tris-EGTA, 5 HEPES HemiNa, pH 7.4) containing 0.4–1 mM MgATP. Patch currents were measured with an Axopatch 200A amplifier (Axon Instruments Inc.) under voltage-clamp mode and were recorded at 1 kHz. For subsequent analysis, records were digitally filtered at 50 Hz and single-channel records were analyzed

using the event detection features of Clampfit 9 (Axon Instruments Inc.).

An apparent open probability (P_o^*) was used as a measure of channel gating. P_o^* was defined as the ratio of NP_o-for-all-levels divided by N, where NP_o-for-all-levels was obtained after single-channel searching using the event detection features in Clampfit 9 and N is the apparent number of channels in a patch.

Reagents

Bischloroethylnitrosourea (BCNU), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), $CuCl_2$, Glutathione (GSH), isoproterenol (Isop) and isobutylmethyl xanthine (IBMX) were obtained from Sigma. $MTSET^+$ ([2-(trimethylammonium)ethyl] methanethiosulfonate bromide) was obtained from Toronto Research Chemicals.

Results

The conductance of oocytes expressing T338C CFTR and the response to 2-ME or DTT were altered by BCNU, an inhibitor of glutathione reductase

GSH is the most abundant free thiol in cells and it has high affinity for metals (Rabenstein 1989). I thus considered the possibility that variable intracellular GSH concentrations might contribute to the variability in initial conductance and responses to 2-ME or DTT seen in oocytes expressing T338C CFTR (Liu et al. 2006) by altering the fractional distribution of channels containing copper. To test this hypothesis, BCNU, an inhibitor of glutathione reductase that is expected to decrease intracellular GSH, was used to perturb intracellular GSH.

Summarized in Fig. 1 are results obtained from oocytes expressing T338C or T338A CFTRs that were either untreated, or exposed to 100 μM BCNU for 72 h prior to electrophysiological recording. Oocytes expressing T338C CFTR and exposed to 100 μM BCNU exhibited a significantly lower initial steady state conductance (22 ± 4 μS) than untreated oocytes (92 ± 14, μS, P-value < 0.05). They also exhibited a larger fractional increase in conductance upon exposure to 1 mM 2-ME (Fig. 1A) (P-value < 0.05). The conductances after exposure to 2-ME were not significantly different between treated (119 ± ((μS) and untreated oocytes expressing T338C CFTR (98 ± 13 μS). The inset to Fig. 1A contains the results obtained from a second population of oocytes in which the untreated oocytes exhibited a spontaneously low initial conductance and a robust response to 2-ME, indicative of adventitious copper binding. In this population, oocyte pre-exposed to BCNU did not differ

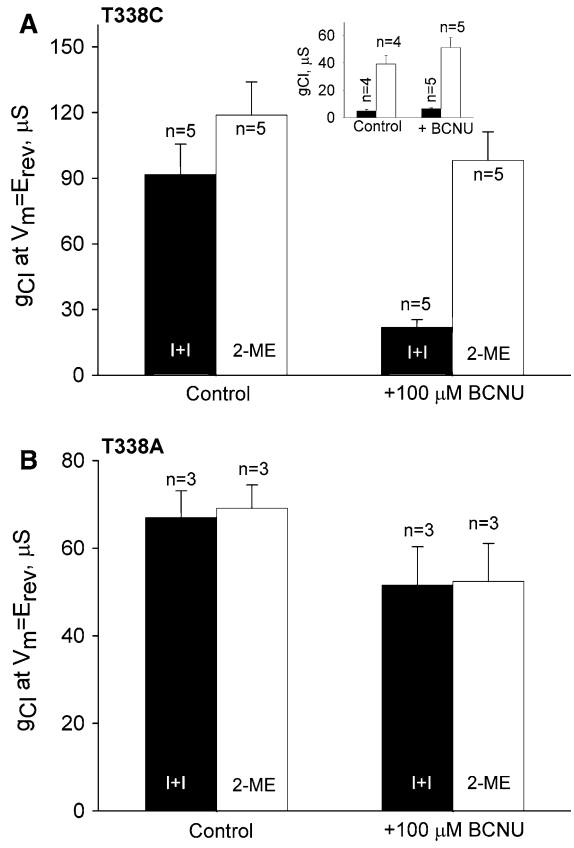

Fig. 1 BCNU altered T338C CFTR conductance and its response to 2-ME. (**A**) The initial steady state conductance of oocytes expressing T338C CFTR (black bars) and the conductance after exposure to 1 mM 2-ME (white bars) were summarized for the control oocytes and oocytes maintained in the incubation solution (MBSH) containing 100 μM BCNU since injection of cRNA. Inset: Results obtained from a different batch of oocytes that were treated and assayed the same way as those shown in panel A. (**B**) The initial steady state conductance of oocytes expressing T338A CFTR (black bars) and the conductance after exposure to 1 mM 2-ME (white bars) were summarized for the control oocytes and oocytes maintained in the storage solution (MBSH) containing 100 μM BCNU since injection of cRNA

significantly from untreated controls, suggesting that the treatment may mimic the "naturally modified" state.

Oocytes expressing T338A CFTR (Fig. 1B) exhibited no response to 2-ME with or without exposure to BCNU. The mean initial conductance of T338A CFTR was slightly lower in BCNU treated oocytes (67 ± 6 μS) than untreated ones (52 ± 9 μS), but the difference was not statistically significant. These results are consistent with the hypothesis that BCNU-induced responses in T338C CFTR are specific to the cysteine at position 338. In addition, the similarity of conductances observed among BCNU treated and untreated oocytes following exposure to 2-ME suggests that the number of channels on the membrane was not markedly altered by BCNU, if at all.

BCNU altered the fractional distribution of single-channel current amplitudes in oocytes expressing T338C CFTR

To determine if BCNU treatment altered open probability or single-channel conductance, I recorded single-channel currents from inside-out patches detached from oocytes expressing T338C CFTR that were either untreated or exposed to BCNU. Several difficulties were encountered in attempts to obtain single-channel records. In oocytes not pre-exposed to 2-ME or DTT, the pattern of distribution of channel amplitudes differed from patch to patch from 0.1 pA to 0.6 pA at pH 7.4. This variability prompted a concern that events with lower current amplitudes could represent the activity of endogenous Cl^- channels that are sometimes seen in oocytes (Liu et al. 2004). To mitigate this potential contamination by non-CFTR channels, a patch was operationally defined as containing T338C CFTR channels if the events were activated by PKA and ATP. Also, channel run down is always a potential confounding factor (Becq et al. 1994).

Summarized in Fig. 2 are fractional distributions of current amplitudes extracted from patches obtained from oocytes expressing T338C CFTR at pH 7.4 (extracellular, $V_m = -100$ mV). In the absence of any treatment (Fig. 2A) 60% (±12) of the events exhibited current amplitudes of about 0.6 pA. We have shown previously that events with 0.6 pA amplitude represent the full conductance of T338C channels at pH 7.4 in the presence of 2-ME or DTT (Liu et al. 2004). The remaining 40% was accounted for by events with current amplitudes in the ranges of 0.1 pA (15% ± 8), 0.2 pA (12% ± 10) and 0.3–0.4 pA (14% ± 6). The noisy appearance of the open state is, at least in part, a reflection of subconductance states demonstrated by Zhang et al. (2005). In oocytes treated with 100 μM BCNU for 72–96 h, the pattern of fractional distribution of current amplitudes was markedly altered (Fig. 2B). Only 28% (±10) of the events exhibited current amplitude in the range of ~0.6 pA (P-value < 0.05). The fractional distribution of events having reduced current amplitudes in the ranges of 0.1 pA, 0.2 pA and 0.3–0.4 pA were: 0.31 ± 0.13, 0.28 ± 0.11 and 0.14 ± 0.07, respectively.

If events with different current amplitudes represent T338C CFTR channels in different chemical states, be it oxidation or metal complexes, some of these channels might be sensitive to DTT, a strong reducing agent and a potent metal ligand (Krezel et al. 2001). Thus, I recorded single-channel currents in oocytes pretreated with 10 mM DTT for 1–24 h (Fig. 2C, D). DTT treatment resulted in a significant *increase* in the fraction of events having ~0.6 pA current amplitude in both BCNU treated (P-value < 0.05) (Fig. 2D) and control conditions (P-value < 0.05) (Fig. 2C). Exposure to DTT also resulted in a corresponding *decrease* in the number of small current amplitude events. After exposure to DTT, events having current amplitude of 0.6 pA accounted for 94%(±3) in control oocytes and 96%(±2) in BCNU-treated oocytes. The simultaneous increase in 0.6 pA channels and decrease in channels with lower amplitudes is consistent with the conversion of channels with lower current amplitudes to channels having current amplitude of 0.6 pA.

Similar experiments were also performed at $pH_{extra} = 6.0$ (Fig. 3). We reported previously that the single-channel conductance of T338C CFTR is larger at pH 6.0 (~9 pS) than at pH 7.4 (Liu et al. 2004). At pH 6.0, the open channel current also appeared less flickery. As shown in Fig. 3B, BCNU and DTT evoked effects similar to that seen at $pH_{extra} = 7.4$ in which pre-treatment of oocytes with BCNU tended to increase the fractional distribution of lower amplitude current events while exposure to DTT tended to decrease the fractional distribution of

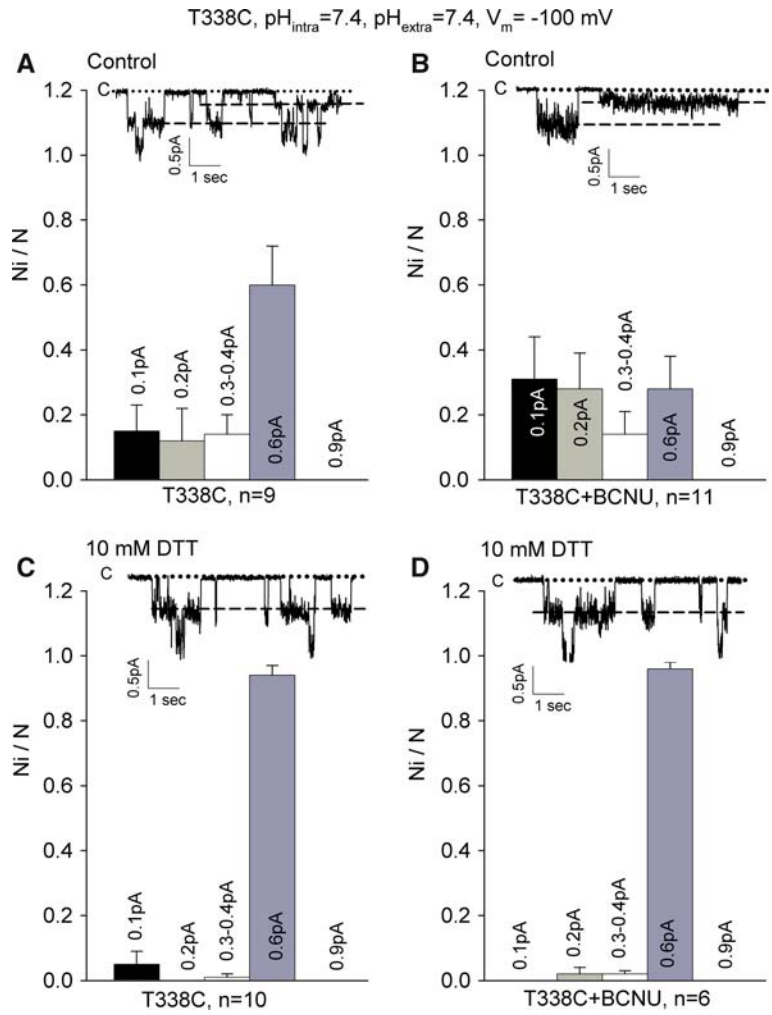

Fig. 2 BCNU altered the fractional distribution of current amplitudes of single T338C CFTR channels at pH 7.4. Fractional distribution of single-channel current amplitudes at pH_{extra} = 7.4 from patches obtained from T338C CFTR expressing oocytes that were: (**A**) incubated in MBSH, (**B**) incubated in MBSH containing 100 μM BCNU since injection of cRNA, (**C**) incubated in MBSH and exposed to 10 mM DTT for about 1 to 24 hours before patching or MBSH, (**D**) incubated in MBSH containing 100 μM BCNU since injection of cRNA and exposed to 10 mM DTT for about 1 to 24 hours before patching. The sample current traces obtained at V_m = –100 mV for each group are shown above the bars

lower amplitude current events (Fig. 3C, D). Also noteworthy is the fact that the fractional distribution of lower amplitude events at pH 6.0 was less than that seen at pH 7.4.

To verify that a cysteine was required for the multiple current amplitudes observed in T338C CFTR, I recorded single-channel currents of T338A CFTR. The single-channel conductance of this construct is greater than that of T338C CFTR (Linsdell et al. 1998; Liu et al. 2004). As shown in Fig. 4, at V_m = –100 mV and pH 7.4 (pipette), with (Fig. 4B) or without (Fig. 4A) previous exposure to BCNU, a majority of the events exhibited a current amplitude of 0.9 pA. Thus exposure to BCNU did not result in any significant change in the fractional distribution of 0.9 pA events in T338A CFTR channels. No difference was detected among the apparent open probabilities (NP_o/N) of T338A CFTR channels (pH 7.4) under control or BCNU treated conditions using records obtained at 500 μM intracellular ATP. In patches from BCNU-treated oocytes, NP_o/N averaged 0.179 ± 0.056 (n = 3 patches) whereas in untreated controls, NP_o/N averaged 0.187 ± 0.041 (n = 3 patches). Similar behavior was seen at pH 6.0 (Fig. 4C, D). These results are consistent with the premise that the effects of both BCNU and DTT required a cysteine at position 338.

Low concentration of GSH reverses sponstanous and copper-modified states at T338C locus

The impact of BCNU on the chemical state of a cysteine at 338 suggests that in the event of reduction

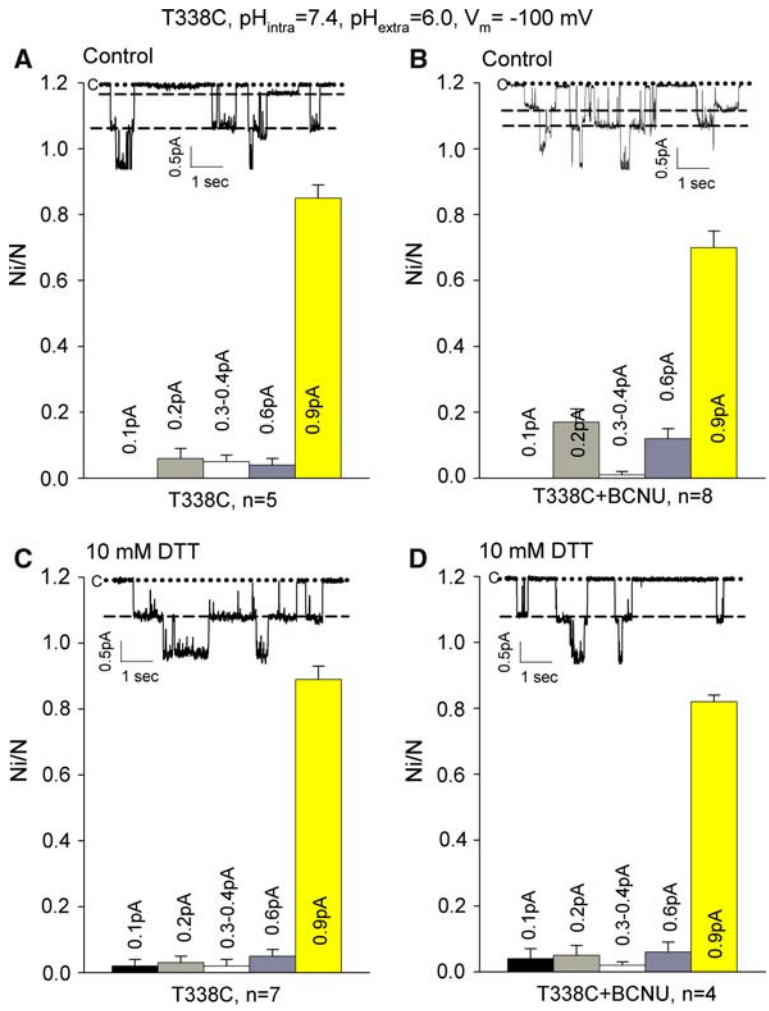

Fig. 3 BCNU altered the fractional distribution of current amplitudes of single T338C CFTR channels at pH 6.0. Fractional distribution of single-channel current amplitudes at pH_{extra} = 6.0 from patches obtained from T338C CFTR expressing oocytes that were: (**A**) incubated in MBSH, (**B**) incubated in MBSH containing 100 μM BCNU since injection of cRNA, (**C**) incubated in MBSH and exposed to 10 mM DTT for about 1 to 24 hours before patching, (**D**) incubated in MBSH containing 100 μM BCNU since injection of cRNA and exposed to 10 mM DTT for about 1 to 24 hours before patching. The sample current traces obtained at V_m = –100 mV for each group are shown above the bars

of cytoplasmic GSH the cysteine at 338 is more likely to be chemically altered, perhaps by coordinating copper. Earlier work (Linsdell and Hanrahan 1998; Kogan et al. 2003) suggest that GSH might actually traverse the CFTR channel or be transported at the expense of ATP hydrolysis. In principle such a process could bring GSH into proximity with position 338 that lies within the anion conduction pathway. Alternatively, efflux of GSH and GSH-conjugates via an endogenous pathway in *Xenopus* oocytes (Ballatori et al. 1996) may lead to a higher local concentration of GSH in the extracellular space between the plasma membrane and the follicular membrane bringing GSH into proximity with T338C.

Because it is impossible at present to assay the intracellular concentration of GSH in intact cells in real time, I chose to use a functional assay to characterize the impact of externally applied GSH on the spontaneously-altered state and copper-modified state of T338C CFTR. A naive oocyte expressing T338C CFTR (Fig. 5A), was first exposed to 1 μM and then to 1 mM GSH, resulting in rapid, dose-dependent increases in conductance, similar to those seen after treatment with DTT or 2-ME, indicating a reversal of the modified state of this engineered cysteine. Upon washout of GSH, the conductance decreased, but exposure to 1 mM DTT increased the conductance to the level prior to GSH washout. The conductance was stable after washout DTT. Afterwards, exposure to 1 mM $CuCl_2$ induced a decrease in conductance and subsequent washout out led to partial reversal in conductance, similar to those reported previously (Liu et al. 2006). Under copper modified condition, exposure to 1 μM GSH and

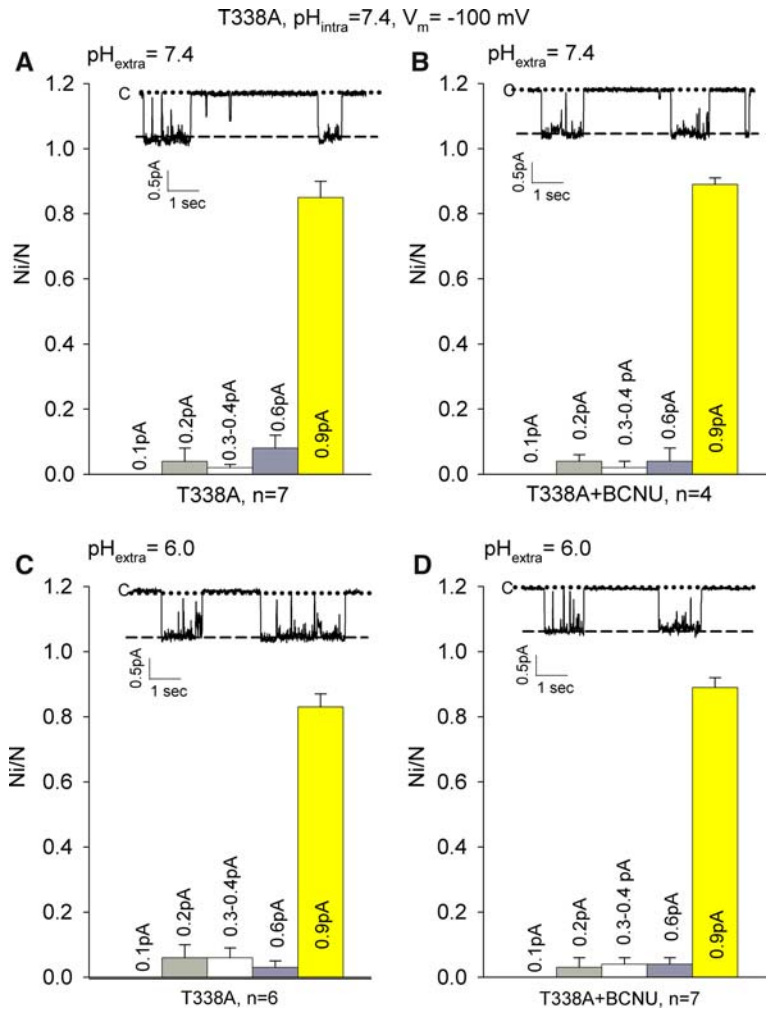

Fig. 4 BCNU had no effect on single T338A CFTR conductance. Fractional distribution of single-channel current amplitudes at pH 7.4 from patches obtained from T338A CFTR expressing oocytes that were: (**A**) incubated in MBSH, (**B**) incubated in MBSH containing 100 μM BCNU since injection of cRNA. Fractional distribution of single-channel current amplitudes at pH 6.0 from patches obtained from T338A CFTR expressing oocytes that were: (**C**) incubated in MBSH, (**D**) incubated in MBSH containing 100 μM BCNU since injection of cRNA. The sample current traces obtained at V_m = –100 mV for each group are shown above the bars

1 mM GSH also resulted in dose-dependent increases in conductance. Conductance also decreased following GSH washout. The similar efficacies of GSH on T338C CFTR conductance under naive and external copper-bound state strongly suggest a similar, if not identical chemical modification of T338C under the two conditions.

The above result suggests that extracellular GSH can perturb copper binding at T338C locus with an affinity in the micromolar range. This behavior is consistent with the high stability constant of GSH-copper complex (Rabenstein 1989). It is noteworthy that following washout of GSH, the subsequent fractional decrease in conductance varied (data not shown). It is not clear at the present what gave rise to this variability. Regardless, a cysteine at position 338 was essential for the GSH effect because at concentrations as high as 10 mM, GSH had no effect on conductance of oocytes expressing Cys-less CFTR (Fig. 5B, n = 2) or T338A CFTR (Fig. 5C, n = 2).

It should be pointed out that GSH exhibited a marked difference in its efficacy to chelate copper and its efficacy to break a mixed disulfide bond between an engineered cysteine and a methanethiol-sulfonate reagent, $MTSET^+$. Results shown in Fig. 6A indicated that GSH could only partially reverse the mixed disulfide bond between T338C and $MTSET^+$ and could do so only at a concentration nearly 1,000 fold higher than that needed to perturb the copper binding site. Similar behavior was observed in R334C CFTR (Fig. 6B). These results indicate that although GSH is capable of breaking a mixed disulfide bond at 338, the reaction precedes at a much lower rate and required a much higher

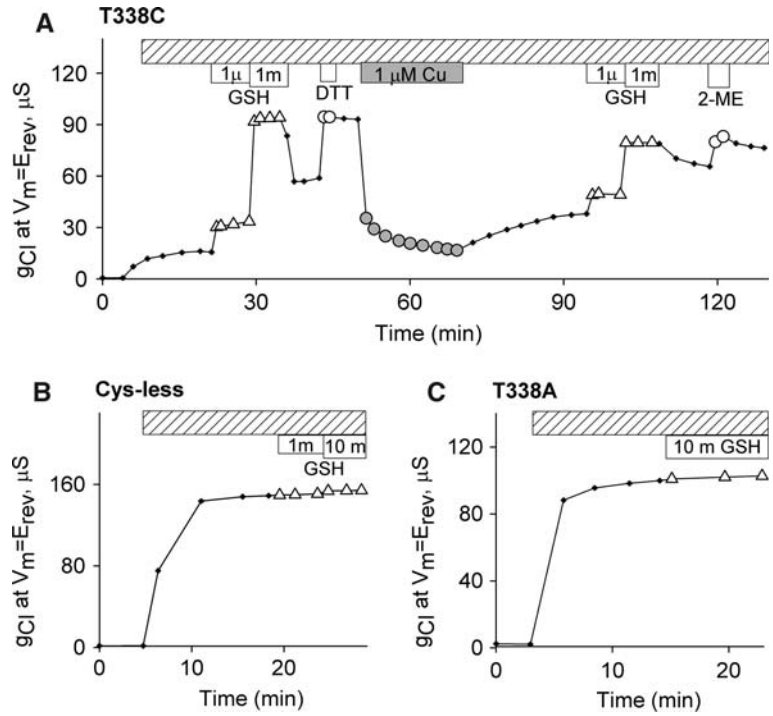

Fig. 5 Extracellular GSH could remove copper from T338C locus. (**A**) Following activation by stimulatory cocktail (10 µM Isop and 1 mM IBMX, hatched bar and crosshair), a naive oocyte expressing T338C CFTR was exposed to: 1 µM and then 1 mM GSH (open triangles), 1 mM DTT (open circles), 1 mM $CuCl_2$ (grey circles), 1 µM GSH and 1 mM GSH, 1 mM 2-ME (open circles), (n = 4). (**B**) Following activation (hatched bar and crosshair), an oocyte expressing Cys-less CFTR was exposed to: 1 mM and then 10 mM GSH (open triangles). (**C**) Following activation (hatched bar and crosshair), an oocyte expressing T338A CFTR was exposed to 10 mM GSH (open triangles)

concentration than that needed for chelating copper. These experiments support the notion that GSH is an importamt determinate of chemical reactivity of T338C in naïve oocytes.

Discussion

We have proposed that an adventitious metal binding site having a high affinity for copper can form at the 338C locus. Although it is not clear when, during the life cycle of the CFTR protein, copper is incorporated into the pore, the current results suggest that intracellular GSH may play a role in determining the nature and extent of the chemical change at position 338 and that the variability in this intracellular pool (Heidemann and Hamborg 1984; Romero et al. 1997) may give rise to the observed variability in initial conductance and variable response to 2-ME or DTT among oocytes. Variable GSH content was reported in different female *Xenopus* laevis (Heidemann and Hamborg 1984). The GSH content of oocytes from three females ranged from 236 to 295 nmol/ml, although GSH level was not significantly different from mature oocytes and immature oocytes from the same female. Cellular GSH content was also found to be variable through out culturing period in V79 cells (Romero et al. 1997). GSH content dropped significantly from 124 nmol/mg protein during the exponential growth phase to 92 nmol/mg protein during confluency (Romero et al. 1997).

GSH is the most abundant free thiol in cells. *Xenopus* oocytes contain about 1.0 mole of GSH and 0.3 mol GSSG per oocyte (Heidemann and Hamborg 1984), which are equivalent to about 2 mM GSH and 0.6 mM GSSG assuming a 0.5 mm radius for an oocyte. In addition, GSH is known to coordinate copper (Rabenstein 1989) and to be an important determinant of the disposition of intracellular copper (Freedman et al. 1989; Ciriolo et al. 1990; Ascone

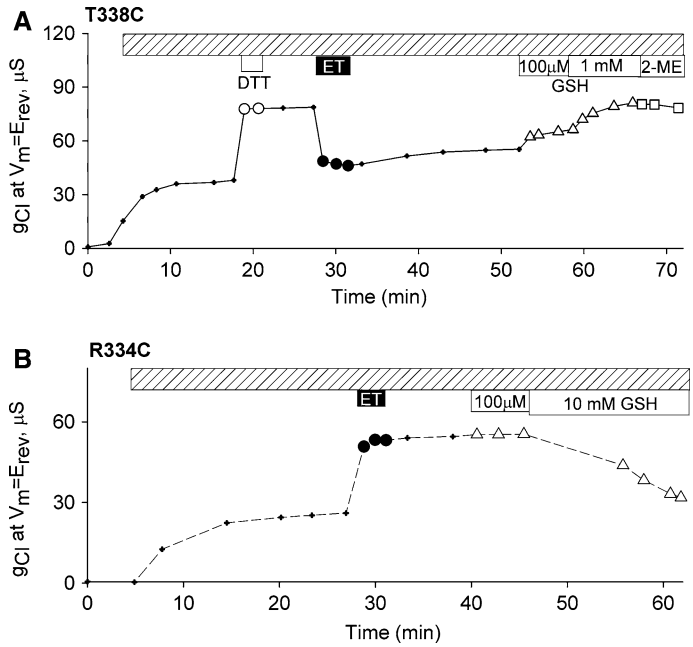

Fig. 6 Breaking a mixed disulfide bond by GSH required a much higher concentration than that needed to chelate copper. (**A**) Following activation by stimulatory cocktail (10 μM Isop and 1 mM IBMX, hatched bar and crosshair), a naive oocyte expressing T338C CFTR was exposed to: 1 mM DTT (open circles), 1 mM $MTSET^+$ (black circles), 100 μM GSH and 1 mM GSH (open triagles), 1 mM 2-ME (open squares). (**B**) Following activation by stimulatory cocktail (10 μM Isop and 1 mM IBMX, hatched bar and crosshair), an oocyte expressing R334C CFTR was exposed to: 1 mM $MTSET^+$ (black circles), 100 μM GSH and 10 mM GSH (open triagles)

et al. 1993; Ferreira et al. 1993). The influence of BCNU on the 2-ME/DTT-sensitive conductance of oocyte expressing T338C CFTR suggests that decreasing cytosolic GSH increases the likelihood that copper will be bound by the adventitious metal center that is inadvertently created in the cysteine-substituted channel.

The concentration of free copper is thought to be vanishingly small in *Xenopus* oocytes (Nomizu et al. 1993) and other cells (Rae et al. 1999; O'Halloran and Culotta 2000; Finney and O'Halloran 2003) due to the presence of GSH and a variety of other copper-binding proteins having copper affinities ranging from zeptomolar to micromolar (Rabenstein 1989; Bethin et al. 1995a; Bethin et al. 1995b; Ohta et al. 2001; Changela et al. 2003; Luk et al. 2003; Palumaa et al. 2004; Sivaraja et al. 2006). Interestingly, a kinetically labile copper pool found mainly in mitochondria and the Golgi apparatus was reported in mouse fibroblast cells (3T3) (Yang et al. 2005). GSH has been shown to play an important role in the incorporation of copper into metalloproteins (Freedman et al. 1989; Ciriolo et al. 1990; Ascone et al. 1993; Ferreira et al. 1993). In a hepatoma cell line (HAC), 60% of cytoplasmic copper was bound to GSH (Freedman et al. 1989). Thus one might expect that a decrease in cytosolic GSH would provoke a redistribution of copper within the cell. In the event of reduced GSH, the possibility of copper binding to the adventitious site during some phase of the life cycle of the protein might be increased. Alternatively, contamination of trace amount of copper in experimental solutions could also contribute to the adventitious copper binding site, although detection of copper contamination is hampered by the fact that the solutions are all chloride based (Liu et al. 2006).

Linsdell and Hanrahan, on the basis of ion substitution studies proposed that GSH and GSSG are capable of entering the CFTR pore from the cytoplasmic side (Linsdell and Hanrahan 1998). Using inside-out vesicles prepared from Sf9 cells expressing CFTR, Kogan et al. (2003) reported significant GSH flux in the presence of MgAMP-PNP that could be attributed to transport through CFTR channels. Permeating GSH

could, in principle, remove bound copper from the adventitious site. A similar role might also be played by extracellular GSH. *Xenopus* oocytes export GSH and GSH conjugates (Ballatori et al. 1996), and it is possible that GSH accumulates near the extracellular space between the plasma membrane and follicular membrane. This local concentration of GSH might be sufficient to alter the coordination geometry or remove copper from the CFTR protein.

The results presented here are consistent with the idea that intracellular GSH may play a role in determining the nature and extent of the chemical change at position 338. The dose-dependent response to extracellular GSH is consistent with an equilibrium mechanism in which increasing GSH shifted the equilibrium towards a state where the copper at the T338C locus was either freed or the coordination geometry was perturbed.

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References

Ascone I, Longo A et al (1993) An X-ray absorption study of the reconstitution process of bovine Cu,Zn superoxide dismutase by Cu(I)-glutathione complex. FEBS Lett 322(2):165–167

Ballatori N, Wang W et al (1996) An endogenous ATP-sensitive glutathione S-conjugate efflux mechanism in Xenopus laevis oocytes. Am J Physiol 270(5 Pt 2):R1156–R1162

Becq F, Jensen TJ et al (1994) Phosphatase inhibitors activate normal and defective CFTR chloride channels. Proc Natl Acad Sci USA 91(19):9160–9164

Bethin KE, Cimato TR et al (1995a) Copper binding to mouse liver S-adenosylhomocysteine hydrolase and the effects of copper on its levels. J Biol Chem 270(35):20703–20711

Bethin KE, Petrovic N et al (1995b) Identification of a major hepatic copper binding protein as S-adenosylhomocysteine hydrolase. J Biol Chem 270(35):20698–20702

Changela A, Chen K et al (2003) Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science 301(5638):1383–1387

Ciriolo MR, Desideri A et al (1990) Reconstitution of Cu,Zn-superoxide dismutase by the Cu(I).glutathione complex. J Biol Chem 265(19):11030–11034

Eklow L, Moldeus P et al (1984) Oxidation of glutathione during hydroperoxide metabolism. A study using isolated hepatocytes and the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea. Eur J Biochem 138(3):459–463

Ferreira AM, Ciriolo MR et al (1993) Copper(I) transfer into metallothionein mediated by glutathione. Biochem J 292(Pt 3):673–676

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300(5621):931–936

Freedman JH, Ciriolo MR et al (1989) The role of glutathione in copper metabolism and toxicity. J Biol Chem 264(10):5598–5605

Heidemann SR, Hamborg MA (1984) The thiol-disulfide balance during maturation of Xenopus laevis oocytes. J Exp Zool 231(1):93–100

Kogan I, Ramjeesingh M et al (2003) CFTR directly mediates nucleotide-regulated glutathione flux. Embo J 22(9):1981–1989

Krezel A, Lesniak W et al (2001) Coordination of heavy metals by dithiothreitol, a commonly used thiol group protectant. J Inorg Biochem 84(1–2):77–88

Linsdell P, Hanrahan JW (1998) Glutathione permeability of CFTR. Am J Physiol 275(1 Pt 1):C323–C326

Linsdell P, Zheng SX et al (1998) Non-pore lining amino acid side chains influence anion selectivity of the human CFTR Cl- channel expressed in mammalian cell lines. J Physiol 512(Pt 1):1–16

Liu X, Alexander C et al (2006) Variable reactivity of an engineered cysteine at position 338 in cystic fibrosis transmembrane conductance regulator reflects different chemical states of the thiol. J Biol Chem 281(12):8275–8285

Liu X, Zhang ZR et al (2004) CFTR: A Cysteine at Position 338 in TM6 Senses a Positive Electrostatic Potential in the Pore. Biophys J 87(6):3826–3241

Luk E, Jensen LT et al (2003) The many highways for intracellular trafficking of metals. J Biol Inorg Chem 8(8):803–809. Epub 2003 Sep 27

Meredith MJ, Reed DJ (1983) Depletion in vitro of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Biochem Pharmacol 32(8):1383–1388

Messana JM, Cieslinski DA et al (1988) Glutathione protects against exogenous oxidant injury to rabbit renal proximal tubules. Am J Physiol 255(5 Pt 2):F874–F884

Nomizu T, Falchuk KH et al (1993) Zinc, iron, and copper contents of Xenopus laevis oocytes and embryos. Mol Reprod Dev 36(4):419–423

O'Halloran TV, Culotta VC (2000) Metallochaperones, an intracellular shuttle service for metal ions. J Biol Chem 275(33):25057–25060

Ohta Y, Shiraishi N et al (2001) Ascorbate-induced high-affinity binding of copper to cytosolic proteins. Biochem Biophys Res Commun 287(4):888–894

Palumaa P, Kangur L et al (2004) Metal-binding mechanism of Cox17, a copper chaperone for cytochrome c oxidase. Biochem J 382(Pt 1):307–314

Rabenstein DL (1989) Metal complexes of glutathione and their biological significance. In: Dolphin D, Poulson R,

Avramovic O (eds) Glutathione: chemical, biochemical and medical aspects. Wiley-Interscience, York, Part A, pp 147–186

Rae TD, Schmidt PJ et al (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284(5415):805–808

Romero FJ, Zukowski D et al (1997) Glutathione content of V79 cells in two- or three-dimensional culture. Am J Physiol 272(5 Pt 1):C1507–C1512

Shinohara K, Tanaka KR (1979) Mechanism of inhibition of red blood cell glutathione reductase activity by BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea). Clin Chim Acta 92(2):147–152

Sivaraja V, Kumar TK et al (2006) Copper binding affinity of S100A13, a key component of the FGF-1 nonclassical copper-dependent release complex. Biophys J 91(5):1832–1843 Epub 2006 Jun 9

Smith SS, Liu X et al (2001) CFTR Covalent and noncovalent modification suggests a role for fixed charges in anion conduction. J Gen Physiol 118(4):407–432

Yang L, McRae R et al (2005) Imaging of the intracellular topography of copper with a fluorescent sensor and by synchrotron X-ray fluorescence microscopy. Proc Natl Acad Sci USA 102(32):11179–11184

Zhang Z, Cui G et al (2005) Determination of the functional unit of the cystic fibrosis transmembrane conductance regulator chloride channel. One polypeptide forms one pore. J Biol Chem 280(1):458–468