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Interplay between Inhibitory Ferric and Stimulatory Curcumin Regulates Phosphorylation-Dependent Human Cystic Fibrosis Transmembrane Conductance Regulator and ΔF508 Activity

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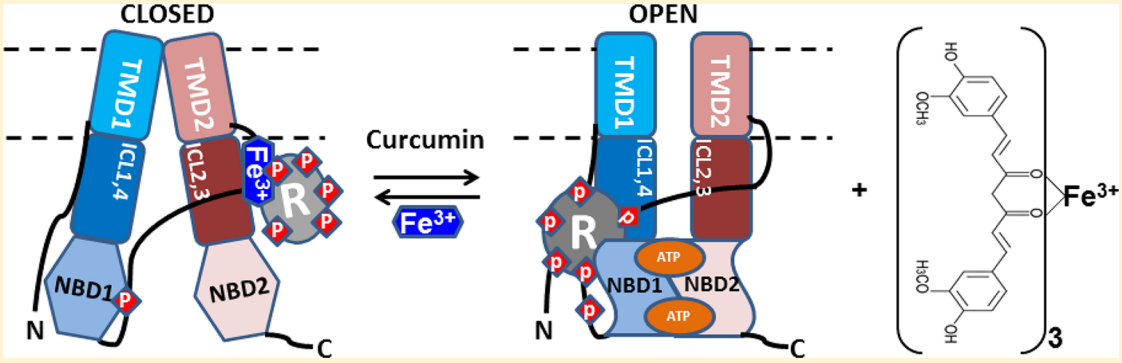

ABSTRACT: Curcumin potentiates cystic fibrosis transmembrane conductance regulator (CFTR) activation in an ATP-independent but phosphorylation-dependent manner. The underlying molecular mechanisms are unclear. Here, HEK-293T cells cultured in an Fe^{3+}-containing medium were transiently transfected with CFTR constructs, and the role of the inhibitory Fe^{3+} bridge between intracellular loop 3 and the regulatory domain of CFTR in this pathway was investigated. The results showed that ethylenediaminetetraacetic acid (EDTA) stimulated phosphorylation-dependent CFTR activation and the stimulation was suppressed by the deletion of the regulatory domain or the insertion of a C832A mutation that removes the Fe^{3+}-binding interface. Furthermore, curcumin potentiation of CFTR was significantly weakened not only by Fe^{3+}-insensitive mutations at the interface between the regulatory domain and intracellular loop 3 but also by *N*-ethylmaleimide or EDTA pretreatment that removes Fe^{3+}. More importantly, potentiation of CFTR was completely suppressed by sufficient Fe^{3+}. Finally, the insertion of Fe^{3+}-insensitive H950R/S768R increased the curcumin-independent activity of ΔF508 but weakened its curcumin potentiation. Thus, Fe^{3+} homeostasis in epithelia may play a critical role in regulating CFTR activity, and targeting Fe^{3+}-chelating potentiators may direct new therapies for cystic fibrosis.

The CFTR Cl^- channel is an important and unique member of the ATP-binding cassette (ABC) superfamily.[1,2] It is canonically activated by ATP-binding-induced dimerization of two cytoplasmic nucleotide-binding domains (NBD1 and NBD2),[3,4] phosphorylation of the unique cytoplasmic regulatory (R) domain by protein kinase A (PKA),[5] and resultant gating inward-to-outward reorientation of two transmembrane domains (TMD1 and TMD2).[6−9] Four intracellular loops (ICLs) extending from TMDs make important interactions with not only NBD1 and NBD2 but also the R domain and thus play a pivotal role in channel gating.[10−17] Although the same ICL1/ICL4−NBD1 and ICL2/ICL3−NBD2 swapping interactions shared by both inward- and outward-facing TMDs facilitate gating regulation by ATP,[6−8,18] three ICL3−R interactions confirmed by disulfide-cross-linking prevent channel opening of human CFTR (hCFTR).[15−17] (1) The H-bond between the OH group of S768 in the R domain and the imidazole group of H950 in ICL3 is responsible for S768 inhibition in the nonphosphorylated state.[16] (2) The state-dependent high-affinity Fe^{3+} bridge between H950 and H954 from ICL3 and C832, D836, H775, and phosphorylated S768 from the R domain is responsible for S768 inhibition in the phosphorylated state.[15] (3) The asymmetric electrostatic attraction between K946 of ICL3 and D835, D836, and E838 of the R domain is responsible for NEG2 inhibition.[17] Because EDTA potentiation of hCFTR activation was not found in Fe^{3+}-insensitive mutants at the ICL3−R interface, endogenous Fe^{3+} was proposed at the ICL3−R interface under physiological conditions.[15] In addition, because *N*-ethylmaleimide (NEM) potentiation of hCFTR

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activity through chemical modification of C832 is tightly coupled to EDTA potentiation, NEM potentiation may result from removal of endogenous and inhibitory Fe^{3+} at the ICL3−R interface.[15]

Cystic fibrosis (CF), the most common fatal recessive hereditary disease, etiologically results from genetic mutation-induced dysfunction of human CFTR at the respiratory epithelium. Two most common CF-causing mutants are G551D and ΔF508. G551D dampens ATP-binding-induced NBD1−NBD2 dimerization while ΔF508 weakens the ICL4−NBD1 interaction.[10,19,20] Thus, an ATP-dependent activation pathway is damaged by two mutations even if the R domain is phosphorylated and a new ATP-independent activation or potentiation pathway is expected to rescue CF-causing mutants. Our recent study demonstrated that K978C/Δ1198 CFTR is still stimulated by PKA without the involvement of NBD2 and the stimulation is suppressed by the deletion of the R domain, strongly suggesting a stimulatory ATP-independent interaction of the R domain with other cytoplasmic domains.[21] Therefore, the PKA-dependent activation pathway can be used to rescue CF-causing mutants with an ATP-dependent gating defect. Until now, only curcumin and FDA-approved VX-770 were found to promote channel opening of G551D and ΔF508 in an ATP-independent but PKA-dependent manner.[22−24] However, the low bioavailability of curcumin prevents its clinical application, while VX-770 has been reported by the European Medicines Agency (EMA) to have a number of off-target effects such as abdominal pain, diarrhea, dizziness, rash, upper respiratory tract reactions, headache, and bacteria in sputum. Therefore, illumination of their PKA-dependent potentiation pathway is necessary for their further medicinal chemistry optimization.

On the other hand, *Pseudomonas aeruginosa* (PA), which chronically infects the lungs of more than 80% of adult patients with CF, requires iron to enhance antibiotic resistance of biofilms grown on human airway epithelial cells. Recent studies indicated that the ΔF508 CFTR mutation promotes PA biofilm formation by increasing Fe^{3+} availability on CF airway cells,[25] while the combined use of tobramycin and FDA-approved Fe^{3+} chelator deferoxamine or deferasirox eliminates PA biofilms.[25,26] More importantly, although a corrector Corr-4a prohibits biofilm formation by increasing the density of ΔF508-mediated chloride secretion in the plasma membrane, it has no effect on the Fe^{3+} concentration in the apical medium.[25] Therefore, Fe^{3+} may promote PA biofilm formation by directly lowering ΔF508 CFTR activity, and deferoxamine or deferasirox may prohibit PA biofilm formation by directly chelating inhibitory Fe^{3+} in ΔF508 CFTR. Because PKA-dependent curcumin potentiation of hCFTR constructs is characterized by an interesting "run-down" (the second curcumin potentiation of phosphorylated hCFTR constructs is weaker than the first after a washout),[22,27] and the chemical structure of both curcumin and VX-770 suggests the potential to chelate Fe^{3+}, investigating a hypothesis that PKA-dependent potentiation of hCFTR and ΔF508 activity results from removal of endogenous inhibitory Fe^{3+} at the ICL3−R interface is pathologically significant. Because curcumin can bind to Fe^{3+} with a high affinity (stability constant, $\log \beta = 22.25$) to form an $Fe(curcumin)_3$ complex,[28,29] it is an ideal template for examining the hypothesis mentioned above. The data show that curcumin may promote PKA-dependent channel activation by removing endogenous inhibitory Fe^{3+} at the ICL3−R interface while sufficient Fe^{3+} suppressed hCFTR potentiation. Accordingly, Fe^{3+} hemeostasis may play a critical role in PA biofilm formation by directly regulating CFTR activity, and an optimal potentiator for treating cystic fibrosis mutants should also be a strong Fe^{3+} chelator.

MATERIALS AND METHODS

Molecular Biology. Wild-type (WT) hCFTR was subcloned into the pCDNA3 mammalian expression vector (Invitrogen). ΔR-S660A CFTR was provided by M. Welsh (University of Iowa, Iowa City, IA).[30] All mutants were produced using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by automated sequencing.

Cell Culture and Transfection. Human embryonic kidney (HEK-293T) cells were cultured in Fe^{3+}-containing Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum and 1 mM penicillin/streptomycin and were transiently transfected with WT or mutant CFTR cDNA using the Lipofectamine transfection kit (Invitrogen). For patch-clamp recordings, the transfected cells were transferred to plastic coverslips and used 1−4 days postseeding. It is noteworthy that CFTR expression varied with time.

Patch-Clamp Analysis. HEK-293T cells expressing CFTR channels were recorded in the inside-out configuration using an Axon 200B amplifier (Axon Instruments, Foster City, CA). This configuration allowed for application of reagents to the cytoplasmic face of the channel protein. CFTR currents were recorded in symmetrical solutions containing 140 mM *N*-methyl-D-glutamine chloride, 3 mM $MgCl_2$, 1 mM EGTA, and 10 mM TES (pH 7.3). The resulting resistance of the borosilicate patch pipette was 3−4 MΩ in the bath solution. Macroscopic currents were evoked using a ramp protocol from 80 to −80 mV and then to 80 mV. Each sweep was taken with a 10.75 s time period and filtered at 200 Hz. Reagents were in turn added to the bath solution. A total of 1.5 mM MgATP and 24 units/mL PKA were usually used to activate the CFTR channel unless otherwise indicated. At steady activation, protein kinase inhibitor (PKI) was usually added to block further phosphorylation to make sure that channel activity could be directly regulated by the subsequent addition of reagents. CFTR blocker glibenclamide (100−200 μM) or CFTR inhibitor $CFTR_{inh172}$ (10 μM) was finally used to suppress the total CFTR currents to evaluate the baseline control current.[31] Several Fe^{3+} chelators, including EGTA, EDTA, and curcumin, were used in this study. Their stability constants for Fe^{3+} are $\sim 10^{20}$, $\sim 10^{25.1}$, and $\sim 10^{22.25}$ M^{-1} at 20 °C, respectively.[28,32] The CFTR stability constant for Fe^{3+} is $\sim 10^{20}$ M^{-1} at 20 °C.[15] Therefore, even when 500 μM $FeCl_3$ was applied to the bath solution, the free Fe^{3+} concentration was still as low as 5×10^{-21} M in the presence of 1 mM EGTA. However, Fe^{3+} can bind to or unbind from CFTR via a ligand exchange between Fe(CFTR) and $Fe(EGTA)^-$, or $Fe(EDTA)^-$ or $Fe(curcumin)_3$.[33] All experiments were conducted at room temperature (22 ± 1 °C).

Statistical Analysis. At least three independent current data were acquired and analyzed using pCLAMP10.2 software (Axon Instruments). Statistical data are presented as means ± the standard error of the mean. Statistical significance was evaluated by a Student's *t* test.

RESULTS

Endogenous Fe^{3+} Inhibits PKA-Dependent hCFTR Activation. A previous study indicated a "run-down" in

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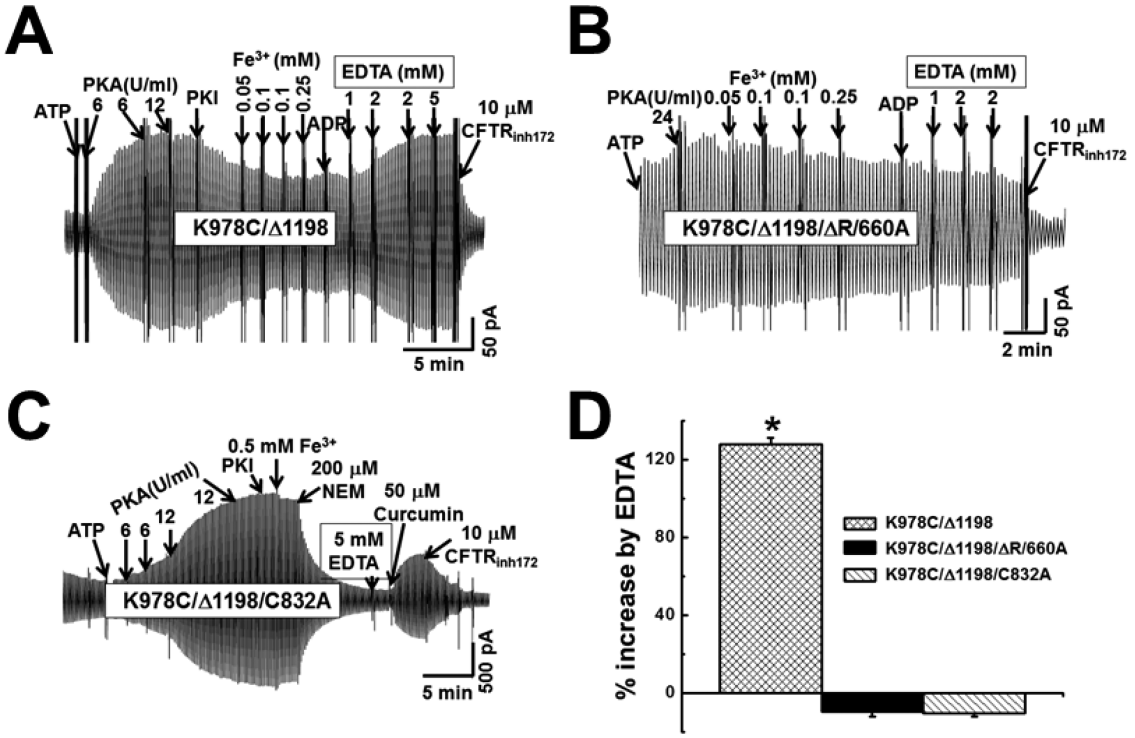

Figure 1. Effects of internal Fe^{3+} and EDTA on hCFTR activity.Inside-out macroscopic currents of hCFTR mutants (A) K978C/Δ1198 CFTR, (B) K978C/Δ1198/ΔR CFTR, and (C) K978C/Δ1198/C832A CFTR, respectively, in response to Fe^{3+} and EDTA. Arrows indicate the time at which each reagent was added. (D) Percentage current changes of CFTR mutants induced by 5 mM EDTA (n = 3; *P < 0.05, from an unpaired Student's t test).

curcumin potentiation of Δ1198 CFTR,[22] suggesting that inhibitory Fe^{3+} may still bind to this CFTR construct even if NBD2 has been deleted. To further examine whether the inhibitory Fe^{3+} bridge between the R domain and ICL3 prevents PKA-dependent channel activation, I investigated the effects of internal Fe^{3+} and EDTA on the current of K978C/Δ1198 CFTR with or without the involvement of the R domain. Figure 1A shows that constitutively active K978C/Δ1198 CFTR was further stimulated not by 1.5 mM ATP but by 24 units/mL PKA. After the stimulation was inhibited by PKI, both internal 500 μM Fe^{3+} and ADP (1.5 mM) had no significant effect on the current. This observation is consistent with our previous report that NBD2 is required for ADP inhibition.[21] To my surprise, a subsequent addition of EDTA (5 mM) dramatically increased the channel current by 129%, and the total current was inhibited by 10 μM $CFTR_{inh172}$.[31] In sharp contrast, the deletion of the R domain, which removes the Fe^{3+}-binding interface, suppressed EDTA potentiation of the K978C/Δ1198 CFTR current (Figure 1B). A similar result was found with the insertion of C832A, which prevents binding of Fe^{3+} to the R–ICL3 interface (Figure 1D). Because K978C/Δ1198/C832A CFTR was not inhibited by PKI (Figure 1C), the channel activity may be saturated so that further EDTA potentiation is impossible. In this case, NEM was used to inhibit the channel current after PKI and Fe^{3+} did not decrease the channel current (Figure 1C). It is interesting that subsequent EDTA (5 mM) application still failed to potentiate the channel current (Figure 1C). Thus, EDTA potentiation, independent of ATP-binding-induced NBD1–NBD2 dimerization, may be due to removal of endogenous inhibitory Fe^{3+} at the ICL3–R interface. In other words, the Fe^{3+} bridge between the R domain and ICL3 may inhibit PKA-dependent CFTR activation by preventing the stimulatory interaction of the R domain with other cytoplasmic parts. Because the effect of Fe^{3+} on K978C/Δ1198 CFTR was weaker than that on WT hCFTR,[15] binding of Fe^{3+} to the ICL3–R interface may be enhanced by the deletion of NBD2.

Fe^{3+}-Insensitive Mutations Weaken Curcumin Potentiation of hCFTR. Because curcumin (50 μM) still increased the channel current of Fe^{3+}- and EDTA-insensitive K978C/Δ1198/C832A CFTR (Figure 1C), curcumin may potentiate hCFTR activation in an Fe^{3+}-independent manner. This proposal is consistent with our previous report that ΔR, Δ1198/ΔR, and K978C/Δ1198/ΔR CFTR constructs are also potentiated by curcumin.[15,16,22] To further test whether Fe^{3+} binding at the ICL3–R interface contributes to curcumin potentiation of hCFTR, I investigated curcumin potentiation with or without Fe^{3+}-insensitive mutations at the ICL3–R interface. Figure 2A shows that the hCFTR current was activated by 1.5 mM ATP and 24 units/mL PKA and inhibited by PKI. However, curcumin (50 μM) dramatically increased hCFTR activity by 260%, and the increases in outward and inward currents were blocked by 100 μM glibenclamide to different extents. In contrast, curcumin potentiation of Fe^{3+}-insensitive H950R activation was significantly weakened (Figure 2B,C). Similar results were seen with other Fe^{3+}-insensitive mutations, including H950A, H954A, H775A, C832A, D836A, S768A, H950A/H954A, and H775A/C832A/D836A (Figure 2C).

NEM or EDTA Pretreatment Weakens Curcumin Potentiation of hCFTR. To exclude the mutation-induced effects on curcumin potentiation of hCFTR, I pretreated the hCFTR with 200 μM NEM to modify C832 and thus to remove Fe^{3+} at the ICL3–R interface.[15] As expected,[34] NEM

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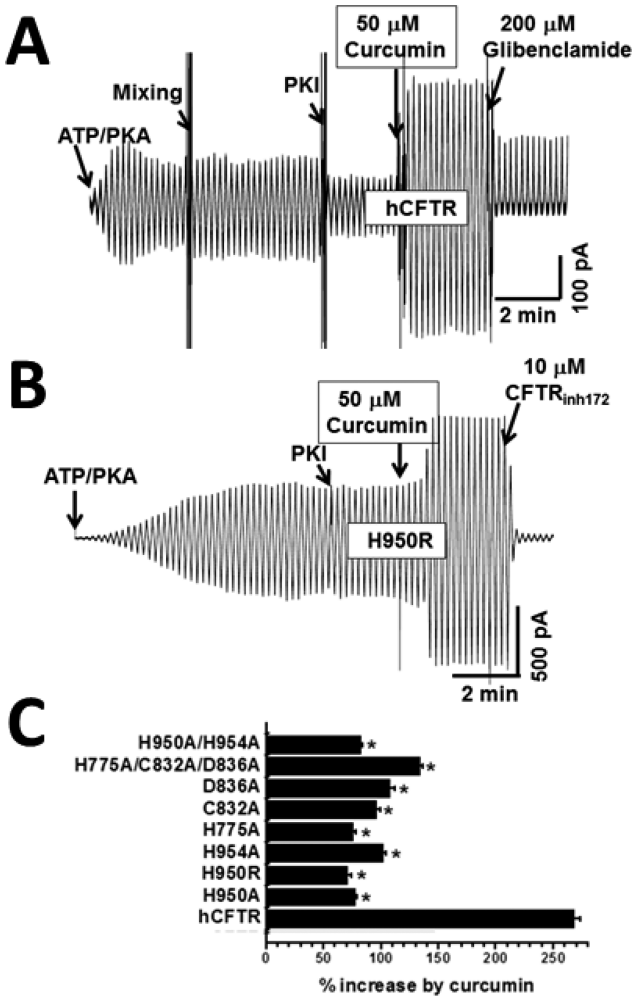

Figure 2. Effects of Fe^{3+}-insensitive mutations on curcumin potentiation of hCFTR activity. Inside-out macroscopic currents of (A) hCFTR and (B) H950R in response to curcumin. Arrows indicate the time at which each reagent was added. (C) Percentage increases in the CFTR current induced by curcumin (n = 3–5; *P < 0.05, from an unpaired Student's t test).

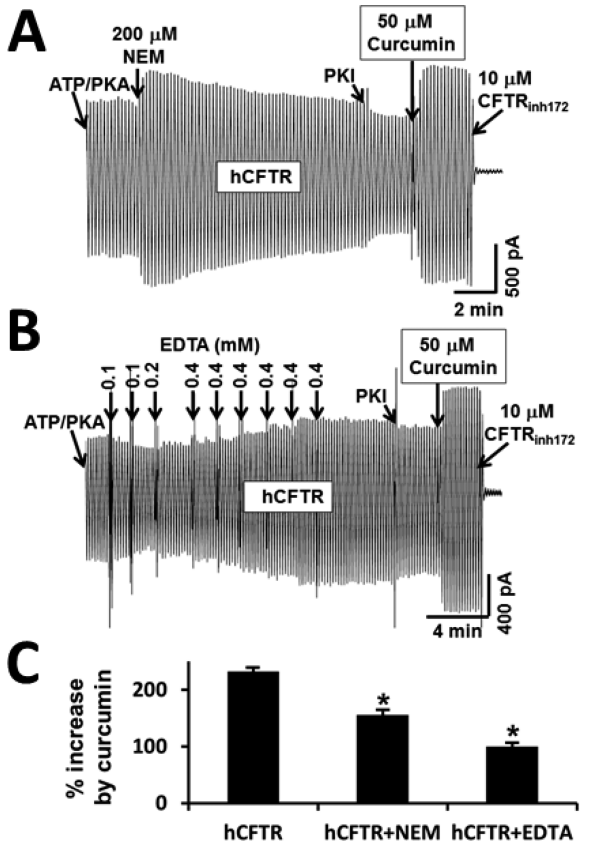

Figure 3. Effects of NEM and EDTA on curcumin potentiation of hCFTR activity. Inside-out macroscopic currents of hCFTR in response to (A) NEM or (B) EDTA and curcumin. Arrows indicate the time at which each reagent was added. (C) Percentage increases in the hCFTR current induced by curcumin (n = 3 or 4; *P < 0.05, from an unpaired Student's t test).

increased channel activity (Figure 3A). After PKI inhibited the channel current, curcumin still potentiated channel activity but with a decreased effect (Figure 3A,C). A similar result was seen after EDTA (1.5 mM) potentiated the channel current to remove Fe^{3+} at the ICL3–R interface (Figure 3B,C).[15] Thus, curcumin potentiation of hCFTR weakened by NEM or EDTA treatment may be due to removal of Fe^{3+} by curcumin. In other words, curcumin potentiation of hCFTR may be Fe^{3+}-dependent. Because the stability constant of curcumin for Fe^{3+} ($\sim 10^{22.25}$ M^{-1}) is smaller than that of EDTA for Fe^{3+} ($\sim 10^{25.1}$ M^{-1})[28,32] but less curcumin than EDTA was required to remove Fe^{3+} at the ICL3–R interface (Figures 2A and 3B), Fe^{3+}-independent curcumin potentiation may promote removal of Fe^{3+} by curcumin.

The $Fe(curcumin)_3$ Complex Fails To Potentiate hCFTR Activity. To further support Fe^{3+}-dependent curcumin potentiation of hCFTR, sufficient Fe^{3+} binding to the ICL3–R interface was expected to inhibit Fe^{3+}-dependent curcumin potentiation but to leave Fe^{3+}-independent curcumin potentiation intact. However, to my surprise, after several doses of Fe^{3+} were in turn added to inhibit hCFTR activity by targeting the ICL3–R interface,[15] subsequent curcumin (50 μM) failed to potentiate any channel activity (Figure 4A,C). To determine why Fe^{3+}-independent curcumin potentiation was also suppressed by Fe^{3+}, I investigated the effect of Fe^{3+} on curcumin potentiation of Fe^{3+}-insensitive C832A. Figure 4B indicates that curcumin (50 μM) potentiated C832A activity, but the potentiation was reversed by a subsequent addition of Fe^{3+} (375 μM). Thus, the suppression of Fe^{3+}-independent curcumin potentiation of hCFTR by Fe^{3+} may result from the formation of fractional $Fe(curcumin)_3$ that may not potentiate CFTR activity possibly because of a large size or a disfavored conformation.

Taken together, these results strongly support the notion that curcumin potentiation of hCFTR may be partially due to removal of inhibitory Fe^{3+} bound at the ICL3–R interface.

Sufficient Fe^{3+} Suppresses NPPB-AM Potentiation of hCFTR. Because of the failure of the $Fe(curcumin)_3$ complex to potentiate hCFTR activity, it is unknown whether binding of Fe^{3+} to the ICL3–R interface directly suppresses Fe^{3+}-independent curcumin potentiation of hCFTR. In this case, I determined the effects of Fe^{3+} on NPPB-AM potentiation. Previous investigation showed that 10 μM NPPB-AM is enough to fully potentiate hCFTR activity even if the R domain or F508 is deleted.[35] Figure 5A shows that 10 μM NPPB-AM increased the hCFTR current by ~78% after the channel had been activated by 1.5 mM ATP and 24 units/mL PKA and pretreated with PKI. The Fe^{3+}-insensitive H950A mutation at the ICL3–R interface failed to change NPPB-AM

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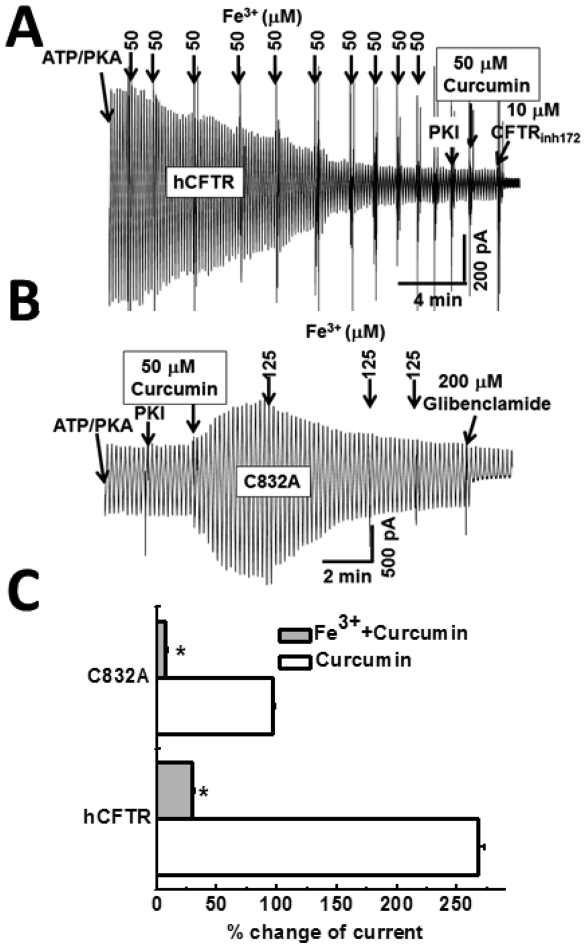

Figure 4. Effects of internal Fe^{3+} on curcumin potentiation. Inside-out macroscopic currents of (A) hCFTR and (B) C832A in response to Fe^{3+} and curcumin. Arrows indicate the time at which each reagent was added. (C) Percentage increases in the hCFTR current induced by curcumin in the presence and absence of Fe^{3+} (≥375 μM) (n = 3 or 4; *P < 0.05, from an unpaired Student's t test). The trace of hCFTR with curcumin is shown in Figure 2A.

potentiation (Figure 5B). Similar results were observed with mutations such as H954A, H775A, and C832A or pretreatment of EDTA (2.4 mM) that prevents binding of Fe^{3+} to the ICL3−R interface (Figure 5D). Therefore, unlike curcumin, NPPB-AM may not chelate Fe^{3+} at the ICL3−R interface while potentiating hCFTR activity. However, after sufficient Fe^{3+} (500 μM) inhibited hCFTR activity, NPPB-AM potentiation was significantly suppressed (Figure 5C,D). Therefore, Fe^{3+} at the ICL3−R interface may directly prohibit potentiation of hCFTR activity.

Fe^{3+}-Dependent Curcumin Potentiation of ΔF508. Previous studies showed that curcumin strongly activated the phosphorylated ΔF508 construct.[27,35] To further examine the Fe^{3+} dependence of curcumin potentiation of ΔF508, I investigated the effect of the Fe^{3+}-insensitive H950R/S768R mutation on curcumin potentiation of ΔF508. Figure 6A shows that ΔF508 activity was very low even in the presence of 1.5 mM ATP and 24 units/mL PKA (Figure 6A,C) but was dramatically potentiated by curcumin upon mixing (Figure 6A,D), in agreement with the previous report.[27,35] In contrast, H950R/S768R/ΔF508 was constitutively active, and 1.5 mM ATP further stimulated mutant channel activation (Figure 6B,C). However, both PKA and curcumin failed to increase its activity (Figure 6B,D). Thus, removal of endogenous and inhibitory Fe^{3+} at the ICL3−R interface by curcumin may promote ΔF508 activation, and thus, the ICL3−R interface may be a potential target for rescuing CF-causing mutants.

DISCUSSION

While several potentiators have been found to rescue the PKA-dependent activity of some CF mutants with an ATP-dependent gating defect, the underlying mechanisms of action are unknown, preventing their further medicinal chemistry optimization.[36] On the other hand, previous study suggested that iron may promote formation of an antibiotic-resistant PA biofilm in the CF lung possibly by inhibiting ΔF508 activity. Therefore, it is necessary to examine if potentiation of hCFTR is Fe^{3+}-dependent. In this study, curcumin, a well-known Fe^{3+} chelator, was employed to test this hypothesis. The results show that endogenous Fe^{3+} at the R−ICL3 interface may inhibit PKA-dependent CFTR activation and potentiation and that removal of endogenous Fe^{3+} by curcumin may promote potentiation of hCFTR and ΔF508 activity. Therefore, an optimal potentiator needs to be a strong Fe^{3+} chelator to release the R domain from ICL3 and thus to promote the stimulatory interaction of the R domain with cytoplasmic domains other than ICL3 (Figure 7).

Release of the R Domain from ICL3 Is Required for PKA-Dependent CFTR Activation. CFTR cannot be activated by ATP only until the R domain is phosphorylated by PKA. Recent NMR structural experiments indicated that the interactions of the isolated nonphosphorylated R domain with isolated NBD1 and NBD2 are weakened upon phosphorylation.[37] This finding is consistent with the notion that R domain phosphorylation releases the R domain from the interface of the NBD1−NBD2 dimer and thus promotes ATP-binding-induced NBD1−NBD2 dimerization, which facilitates channel opening.[3,4,38] Several groups reported that the main inhibitory PKA site is S768.[39−41] Our recent studies further demonstrated that the inhibitory interactions at the ICL3−R interface play a critical role in PKA-dependent CFTR activation. First, as the disruption of the H-bond between the OH group of S768 of the R domain and the imidazole group of H950 promotes ATP-dependent channel activation,[16] the H-bond at the ICL3−R interface may prohibit ATP-binding-induced NBD1−NBD2 dimerization for channel opening. In other words, S768 must be phosphorylated to release the R domain from ICL3 for ATP-gated CFTR activation by disrupting the interfacial H-bond. In addition, ATP cannot activate H950D/S768D unless EDTA is added to remove endogenous Fe^{3+} at the ICL3−R interface.[16] Therefore, Fe^{3+} at the ICL3−R interface may also inhibit CFTR activation by preventing ATP-binding-induced NBD1−NBD2 dimerization (Figure 7). In other words, Fe^{3+} at the ICL3−R interface must be removed to release the R domain from ICL3 for ATP-gated channel activation.

On the other hand, Naren and co-workers revealed a stimulatory interaction between the N-terminal cytoplasmic tail of CFTR and a N-terminal fragment (residues 595−740) of the R domain.[42] Because the stimulation of constitutively active K978C/Δ1198 CFTR by PKA is suppressed by the deletion of the R domain, this stimulatory interaction of the R domain with the N-terminal tail may be independent of NBD1−NBD2 dimerization.[15] In this study, EDTA potentiation of K978C/Δ1198 CFTR was prohibited by the deletion of the R domain or the insertion of C832A that prevents binding of Fe^{3+} to the

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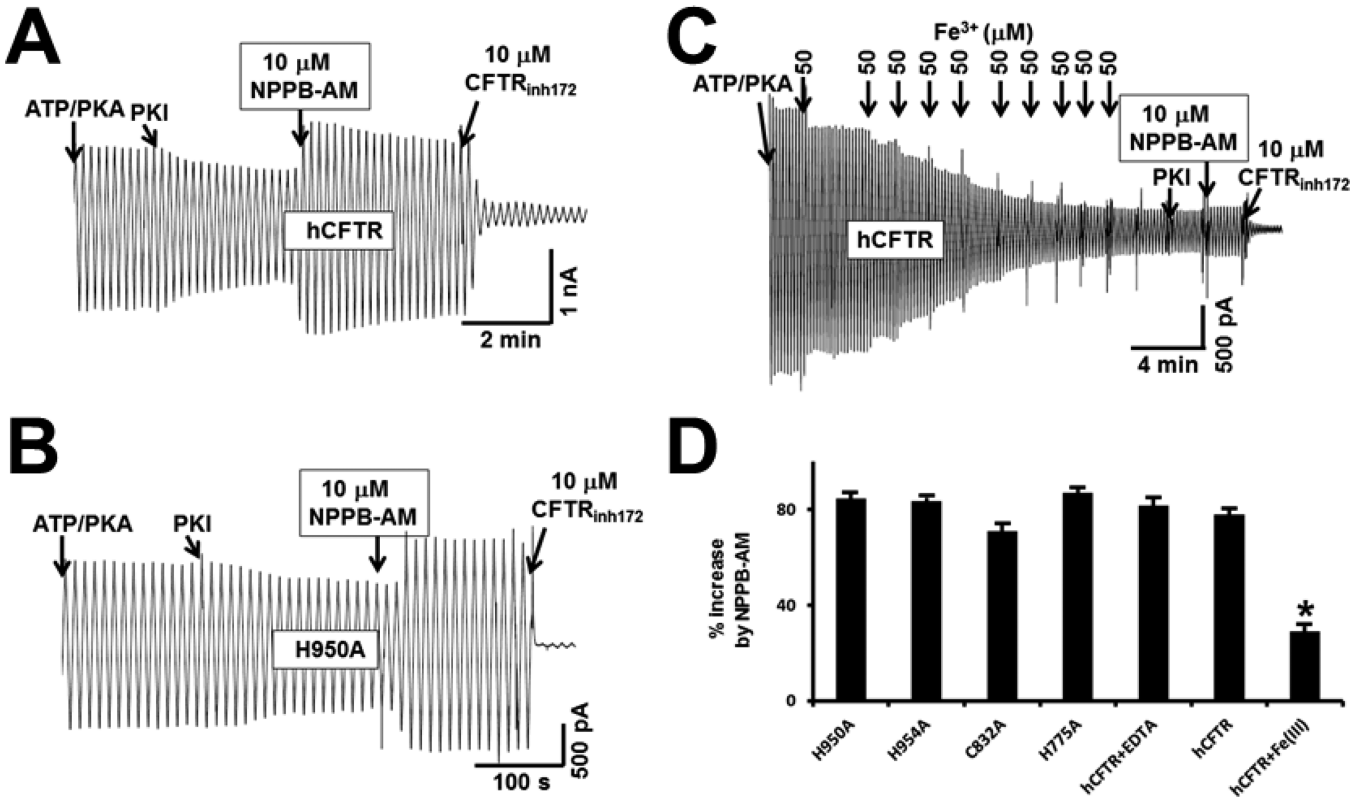

Figure 5. Effects of internal Fe^{3+} on NPPB-AM potentiation. Inside-out macroscopic currents of (A and C) hCFTR and (B) H950A in response to NPPB-AM (A and B) without or (C) with Fe^{3+} pretreatment. Arrows indicate the time at which each reagent was added. (D) Percentage increases in the hCFTR current induced by NPPB-AM (n = 3 or 4; *P < 0.05, from an unpaired Student's t test).

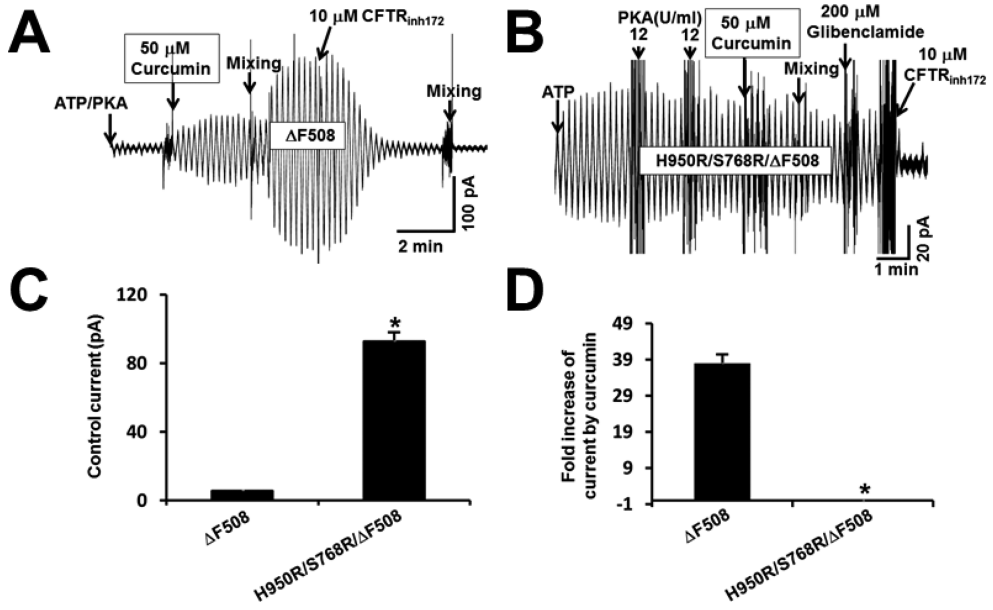

Figure 6. Effects of curcumin on ΔF508 CFTR activity. Inside-out macroscopic currents of hCFTR mutants (A) ΔF508 CFTR and (B) H950R/S768R/ΔF508 CFTR in response to ATP, PKA, and curcumin. Arrows indicate the time at which each reagent was added. (C) Curcumin-independent current triggered by ATP (1.5 mM) and PKA (24 units/mL) (n = 3; *P < 0.05, from an unpaired Student's t test). (D) Fold increases in current by curcumin (n = 3; *P < 0.05, from an unpaired Student's t test).

ICL3−R interface (Figure 1). Thus, the inhibition of CFTR by endogenous Fe^{3+} may also be independent of ATP-binding-induced NBD1−NBD2 dimerization. Therefore, these observations strongly support the notion that endogenous Fe^{3+} at the ICL3−R interface may also inhibit PKA-dependent hCFTR opening possibly by preventing the stimulatory interaction of the phosphorylated R domain with the N-terminal cytoplasmic tail or cytoplasmic parts other than ICL3 (Figure 7). In other words, Fe^{3+} at the ICL3−R interface must be removed to release the R domain from ICL3 for PKA-dependent channel

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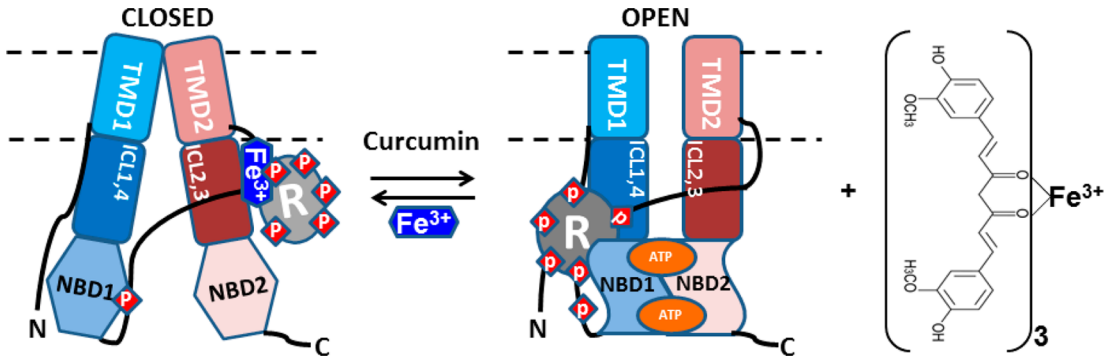

Figure 7. Working model for Fe^{3+}-dependent curcumin potentiation of hCFTR activation. Fe^{3+} binds at the ICL3−R interface with a high affinity. The Fe^{3+} bridge may inhibit hCFTR activation possibly by preventing ATP-binding-induced NBD1−NBD2 dimerization[16] and the stimulatory interaction of the R domain with other ICLs.[15] Curcumin removes Fe^{3+} and thus promotes CFTR opening. However, the $Fe(curcumin)_3$ complex cannot potentiate CFTR activation.

activation by promoting the stimulation of the R domain with cytoplasmic parts other than ICL3 (Figure 1).

Release of the R Domain from ICL3 Is Required for PKA-Dependent CFTR Potentiation. My previous study demonstrated that the H-bond between S768 and H950 prohibits ATP-dependent curcumin potentiation.[16] Thus, phosphorylation of S768 is critical for PKA-dependent potentiation of hCFTR by disrupting the H-bond and thus releasing the R domain from ICL3. In this study, removal of endogenous and inhibitory Fe^{3+} at the ICL3−R interface by curcumin partially potentiated CFTR activation (Figures 2 and 3), and binding of Fe^{3+} to the ICL3−R interface prevented NPPB-AM potentiation (Figure 5). Therefore, removal of interfacial Fe^{3+} is further required for release of the phosphorylated R domain from ICL3, which promotes PKA-dependent CFTR potentiation. Supporting this proposal, previous studies indicated that the second curcumin potentiation of phosphorylated hCFTR, G551D CFTR, or Δ1198 CFTR is weakened after the channel is initially potentiated by curcumin and then washed out.[22,27] It is likely that the first curcumin potentiation may include Fe^{3+}-dependent and Fe^{3+}-independent ones while the second may be Fe^{3+}-independent only after the channel is washed out. Because G551D and Δ1198 mutations prevent ATP-binding-induced NBD1−NBD2 dimerization, Fe^{3+}-dependent curcumin potentiation may be independent of NBD1−NBD2 dimerization. The proposal of endogenous inhibitory Fe^{3+} at the ICL3−R interface may also account for why not NPPB-AM but NPPB can potentiate G551D activity (Figure 5).[35] NPPB with a COOH group and a nearby NH group may have the potential to remove Fe^{3+}, but NPPB-AM may not. Accordingly, removal of endogenous inhibitory Fe^{3+} by curcumin or VX-770 may enhance their potentiation efficacy.

Until now, not NPPB-AM but curcumin and VX-770 were reported to potentiate the channel activity of ΔNBD2 CFTR.[22,24] Because the deletion of NBD2 may enhance binding of Fe^{3+} to the ICL3−R interface (Figure 1), PKA-dependent NPPB-AM potentiation of Δ1198 CFTR may be suppressed by the Fe^{3+} bridge at the ICL3−R interface. On the other hand, both curcumin and VX-770 may remove endogenous inhibitory Fe^{3+} at the ICL3−R interface to promote potentiation of Δ1198 CFTR. In other words, Fe^{3+} at the ICL3−R interface may play a critical role in the ATP-independent but PKA-dependent channel potentiation pathway.

Role of Iron in CF Treatment. Dysfunction of CFTR causes many adult CF patients to be chronically infected with PA in the CF lung. The resultant formation of biofilms enhances antibiotic resistance of PA via their acquisition of iron. It has been reported that the ΔF508 mutation promotes the exuberant formation of antibiotic-resistant biofilms by increasing the amount of release of iron by human airway epithelial cells.[25] Although Corr-4a increases the density of ΔF508-mediated chloride secretion in the plasma membrane and inhibits biofilm formation, it fails to alter the iron concentration in the apical medium.[25] Thus, the low activity of ΔF508 as a result of inhibitory Fe^{3+} at the ICL3−R interface may play a critical role in biofilm formation of human airway epithelial cells. This study further indicated that curcumin potentiation of ΔF508 may be Fe^{3+}-dependent (Figure 6). Because NPPB-AM can also potentiate ΔF508 activity,[22] only fractional curcumin potentiation may result from removal of Fe^{3+} at the ICL3−R interface. Therefore, limiting iron acquisition may be an effective therapeutic strategy for preventing biofilm formation by increasing ΔF508 activity. Supporting this proposal, the combined use of tobramycin and FDA-approved iron-chelating deferoxamine or deferasirox eliminates PA biofilms on CF airway cells overexpressing ΔF508.[26] Further examination of whether these two iron chelators can directly potentiate the activity of CF mutants by removing inhibitory Fe^{3+} at the ICL3−R interface is needed. On the other hand, it is necessary to determine whether curcumin and FDA-approved ivacaftor (VX-770) prevent formation of PA biofilms by directly removing Fe^{3+} at the ICL3−R interface. In addition, because ΔF508 CFTR also has folding and processing problems, an ideal therapy would be a single drug that would be not only a potentiator but also a corrector and an Fe^{3+} chelator without off-target effects but with high efficacy and potency.

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■ ABBREVIATIONS

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; hCFTR, human CFTR; ABC, ATP-binding cassette; R, regulatory; TMD, transmembrane domain; NBD, nucleotide-binding domain; PKA, protein kinase A; PKI, protein kinase inhibitory peptide; ICL, intracellular loop; FDA, Food and Drug Administration; EMA, European Medicines Agency; PA, *P. aeruginosa*; NPPB-AM, 5-nitro-2-(3-phenylpropylamino)benzamide; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate.

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