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Molecular Basis for Fe(III)-Independent Curcumin Potentiation of Cystic Fibrosis Transmembrane Conductance Regulator Activity

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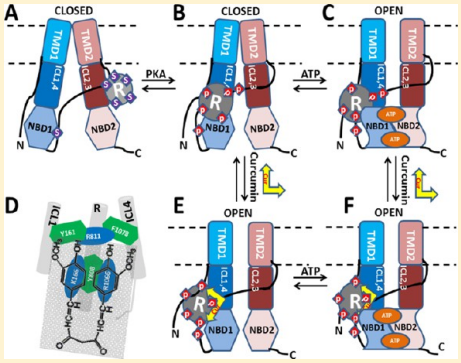
ABSTRACT: Curcumin potentiates the phosphorylation-dependent activity of human cystic fibrosis transmembrane conductance regulator (CFTR) in Fe^{3+}-dependent and Fe^{3+}-independent manners. Although the Fe^{3+}-dependent curcumin potentiation results from removal of endogenous inhibitory Fe^{3+} at the interface of the regulatory (R) domain and intracellular loop (ICL) 3, the molecular mechanism of Fe^{3+}-independent curcumin potentiation is still unknown. Here, HEK-293T cells cultured in an Fe^{3+}-containing medium were transiently transfected with CFTR constructs, and the role of a highly conserved ICL1/ICL4 interface and a stimulatory phosphorylation site S795 or S813 at the C-terminus of the R domain in this potentiation pathway was investigated. The results showed that highly conserved aromatic and positively charged residues at the ICL1/ICL4 interface and phosphorylation site S813 were sensitive to curcumin regardless of whether Fe^{3+} and nucleotide-binding domain 2 were removed. More importantly, spontaneous disulfide cross-linking between curcumin-sensitive ICL1 and S795 was observed to be enough to promote channel opening as curcumin did. Therefore, the phosphorylated R domain, once released from ICL3, may function as a length- and gating-regulatory cross-linker between two transmembrane domains to promote the stimulatory interactions between the R domain and the ICL1/ICL4 interface. Curcumin may potentiate CFTR activity not only by removing inhibitory Fe^{3+} to release the R domain from ICL3 but also by stabilizing the stimulatory R−ICL1/ICL4 interactions. Therefore, the future potentiators with two linked aromatic rings could utilize this potentiation pathway to bypass the need for ATP to rescue the activity of cystic fibrosis mutants with an ATP-dependent gating defect.



CFTR is a member of the ATP-binding cassette (ABC) transporter subfamily but serves as a cAMP-dependent chloride channel with a unique cytoplasmic regulatory (R) domain (residues 646−838) flanked by two large transmembrane domains (TMD1 and TMD2) and two intracellular nucleotide-binding domains (NBD1 and NBD2).[1] Each TMD consists of six transmembrane (TM) helical segments that are probably extended to four intracellular loops (ICLs). CFTR is gated by an reorientation of TMDs between an "inward-facing" conformation and an "outward-facing" one (Figure 1A).[2−6] Because both conformations share the same ICL1/ICL4−NBD1 and ICL2/ICL3−NBD2 swapping interactions (Figure 1A),[2−4,6] NBD1−NBD2 dimerization and separation, induced by ATP binding and hydrolysis at their interface, respectively, can trigger a gating cycle of the channel with a front half (residues 1−633) and a back half (residues 837−1480) only.[7] However, the presence of the R domain prevents full length CFTR from opening until it is phosphorylated by protein kinase A (PKA).[8,9] Therefore, R domain phosphorylation takes a central role in channel activity regulation of native CFTR.

The unphosphorylated R domain, which is much longer than required to bridge the distance between NBD1 and TMD2, is canonically regarded as an inhibitor that sterically prevents ATP-binding-induced NBD1−NBD2 dimerization by interacting with NBD1.[7,9−17] Once phosphorylated by PKA, the regulatory extension (residues 654−679) of the R domain releases from NBD1 for channel opening by NBD1−NBD2 dimerization.[17,18] However, PKA can regulate ATP-independent gating in CFTR constructs without NBD2[19,20] or with G551D, which disrupts one of the ABC signature sequences that line the ATP-binding pocket (site 2).[21] Our recent studies also clearly demonstrated that constitutively active K978C/Δ1198 CFTR is further stimulated by PKA and removal of the segment (residues 708−835) of the R domain suppresses this additional PKA stimulation.[22] Thus, the segment (residues 708−835) of the R domain may activate CFTR by directly acting on the cytoplasmic domains, independent of ATP binding and resultant NBD1−NBD2 dimerization. This proposal is consistent with the observation that phosphorylation promotes binding of the R domain to the rest of

Received: March 2, 2015
Revised: April 12, 2015
Published: April 13, 2015

DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840

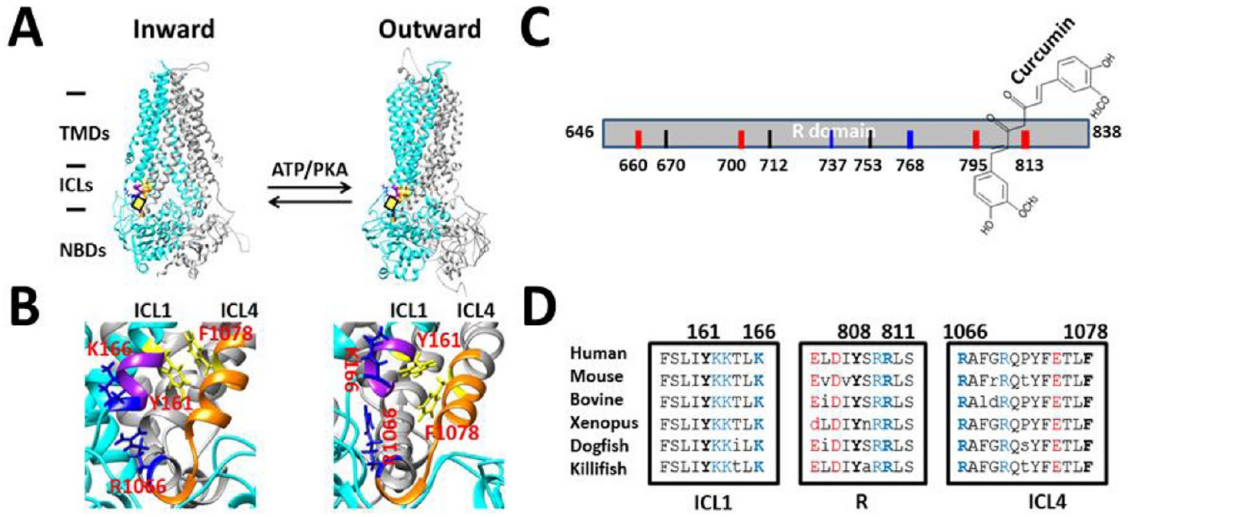


Figure 1. Putative curcumin-binding site in CFTR. (A) Highly conserved interface between ICL1 and ICL4 in outward-facing (right) and inward-facing (left) TMDs based on the crystal structures of bacterial transporters Sav1866[2] and TM287-TM288,[6] respectively. ATP is not shown (right). A putative curcumin-binding site is marked as a yellow block at the ICL1/ICL4 interface. (B) Highly conserved aromatic and positively charged residues at the ICL1/ICL4 interface in the inward- (left) and outward-facing (right) conformations. (C) Curcumin and PKA sites of the R domain with some stimulatory (red) and others inhibitory (blue). (D) Sequence alignment of ICL1, ICL4, and C-terminal PKA site S813 of the R domain of the indicated CFTR species. Potential curcumin-sensitive residues are highlighted.

CFTR.[23,24] Because the electron microscopy (EM) study indicated that the R domain is near the ICLs[5,25] and my recent studies demonstrated that disruption of the interactions of ICL3 with the R domain is not enough to fully activate the CFTR channel,[26] the binding of the R domain to ICLs other than ICL3 is necessary. Because the GST−R domain fusion protein can bind to ICL1 and ICL4[27] and the highly conserved ICL1/ICL4 interface is shared by the inward- and outward-facing TMDs (Figure 1A,B), it is hypothesized that phosphorylation of CFTR may release the flexible R domain from ICL3 and NBD1 for binding to the ICL1/ICL4 interface for optimal channel opening.

On the other hand, curcumin, a natural compound in turmeric, was found to potentiate phosphorylated CFTR activity without ATP or NBD2 necessary as PKA stimulates K978C/Δ1198 activity.[19,22] My recent studies demonstrated that Fe^{3+} prevents channel opening by binding to H950 and H954 of ICL3, and H775, C832, D836, and phosphorylated S768 of the R domain.[28] Removal of endogenous inhibitory Fe^{3+} by curcumin only partially accounts for PKA-dependent curcumin potentiation, while sufficient Fe^{3+} suppresses curcumin potentiation.[29] More importantly, the curcumin potentiation of Fe^{3+}-insensitive C832A can be reversed by sufficient Fe^{3+}, and NEM modification of endogenous accessible cysteines in human CFTR (hCFTR) cannot completely prohibit curcumin potentiation.[29] Therefore, Fe^{3+}-independent curcumin potentiation may not be due to covalent modification of hCFTR by curcumin. Herein, it is further hypothesized that curcumin may promote Fe^{3+}-independent potentiation by stabilizing the putative stimulatory interactions of the phosphorylated R domain with the ICL1/ICL4 interface. Because isoxazolcurcumin, a cyclic curcumin derivative, which lacks the reactive β-diketone moiety and the dimerization effect on CFTR, can still potentiate the channel activity of wild-type (WT) CFTR or a CFTR/ΔNBD2 construct,[30] the β-diketone moiety may not be necessary for Fe^{3+}-independent curcumin potentiation, although it is important for Fe^{3+}-dependent curcumin potentiation.[29] However, curcumin still has two linked aromatic rings to form a $\pi-\pi$ interaction with an aromatic residue or to form a cation$-\pi$ interaction with a positively charged residue.[31] Thus, the curcumin sensitivity of several highly conserved aromatic and positively charged residues at the highly conserved ICL1/ICL4 interface and the PKA site of the R domain (Figure 1B−D) was determined, and the effects of both disulfide cross-linking of ICL1 to the R domain and curcumin on channel gating were further compared in this study. The results support the hypotheses mentioned above.

MATERIALS AND METHODS

Molecular Biology. WT human CFTR (hCFTR) and mouse CFTR (mCFTR) were subcloned into the pCDNA3 mammalian expression vector (Invitrogen). All mutants were produced using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by automated sequencing. V510A was used to increase the level of expression of Cys-free CFTR mutants.[32]

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 at 37 °C under a 5% CO_2 atmosphere and were transiently transfected with WT or mutant CFTR cDNA using the Lipofectamine transfection kit (Invitrogen). CFTR expression in the transfected HEK-293T cells was verified by immunoblotting. Cells expressing processing mutants such as F1078A and R1066H were grown for 1−2 days at 27 °C and then at 37 °C to promote protein expression. For patch-clamp recordings, the transfected cells were transferred to plastic coverslips and used 1−4 days postseeding.

Patch-Clamp Analysis. HEK-293T cells expressing CFTR channels were recorded in the inside-out or whole-cell configuration using an Axon 200B amplifier (Axon Instruments, Foster City, CA). 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

DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840

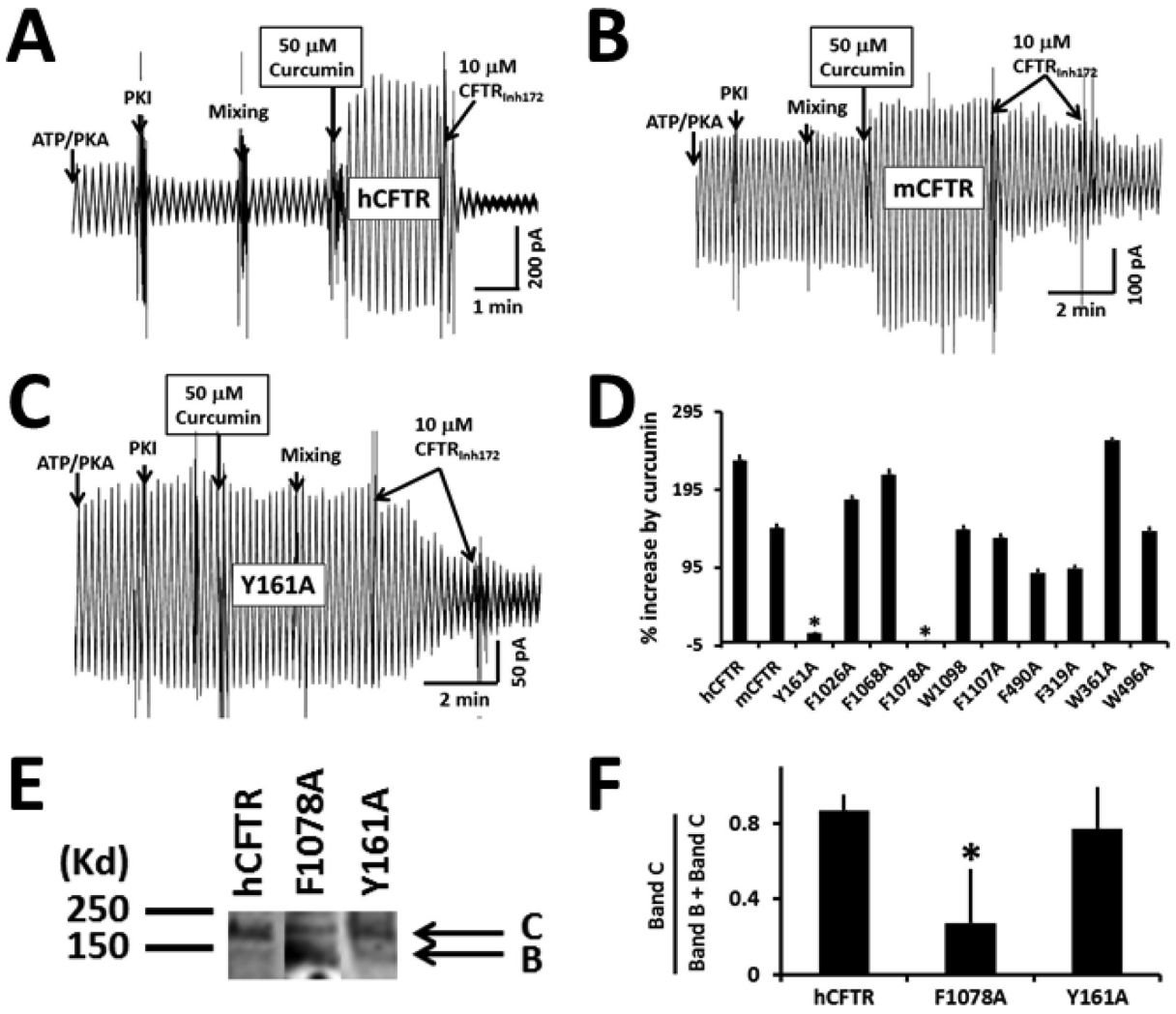


Figure 2. Effects of aromatic residues at the ICL1/ICL4 interface on curcumin potentiation and CFTR expression. Inside-out macroscopic currents of (A) WT hCFTR and (B) mCFTR and (C) Y161A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA. Further phosphorylation was blocked by the addition of PKI (1.4 μg/mL). $CFTR_{inh172}$ (10−20 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. (D) Relative curcumin-induced current potentiation (n = 3−5; *P < 0.001, from an unpaired Student's t test). (E) Immunoblots of WT hCFTR and its mutants. (F) Relative intensities of mature band C of WT hCFTR and its mutants (n = 3−7; *p < 0.05, from an unpaired Student's t test).

resulting resistance of the borosilicate patch pipette was 3−4 MΩ in the bath solution. Inside-out and whole-cell macroscopic currents were evoked using a ramp protocol from 80 to −80 mV and then to 80 mV or from 20 (or 40) mV to −20 (or −40) mV and then to 20 (or 40) mV, respectively. Each sweep was taken with a 10.75 s time period and filtered at 200 Hz. Single-channel recordings at −60 mV were filtered at 20 Hz. Reagents were in turn added to the bath solution. A total of 1.5 mM MgATP and 24−48 units/mL PKA were used to activate the CFTR channel from an inside-out patch, while 10−50 μM forskolin was used to evoke the whole-cell CFTR current. Previous studies demonstrated that both external and internal curcumin can potentiate CFTR activity.[19,22,26,29,33−35] At steady activation, internal protein kinase inhibitor (PKI) (1.4 μg/mL) was usually added to block further phosphorylation to ensure that channel activity could be directly regulated by the subsequent addition of reagents. A voltage-dependent blocker glibenclamide (100−200 μM) or CFTR inhibitor $CFTR_{inh172}$ (5−20 μM) was finally used to evaluate the CFTR-mediated current.[36] Single-channel open probabilities (P_o) were calculated assuming that the number of channels per patch equals the maximal detected number of simultaneous openings. All experiments were conducted at room temperature (22 ± 1 °C).

Western Blot Analysis. Transfected HEK-293T cells expressing hCFTR WT and mutants were washed in divalent-free PBS (Mediatech, Herndon, VA) and then solubilized in 100 μL of sodium dodecyl sulfate (SDS) sample buffer for SDS−polyacrylamide gel electrophoresis (SDS−PAGE) analysis. Each sample was running on a 4 to 15% SDS PAGE gel. Separated proteins were transferred onto PVDF membranes for 60 min at 24 V (Genie blotter, Research Products International Corp.). The membranes were blocked overnight with LI-COR blocking buffer (LI-COR, Lincoln, NE) and then Western-blotted with the C-terminal CFTR antibody mAb 24-1 (R&D Systems) at a 1:5000 dilution and detected with goat anti-mouse Alexa Fluor 680-conjugated antibody (Molecular Probes, Eugene, OR) at a 1:100000 dilution. Blots were extensively washed by a TBS buffer and then scanned to obtain fluorescent images with an Odyssey scanner (LI-COR).

DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840

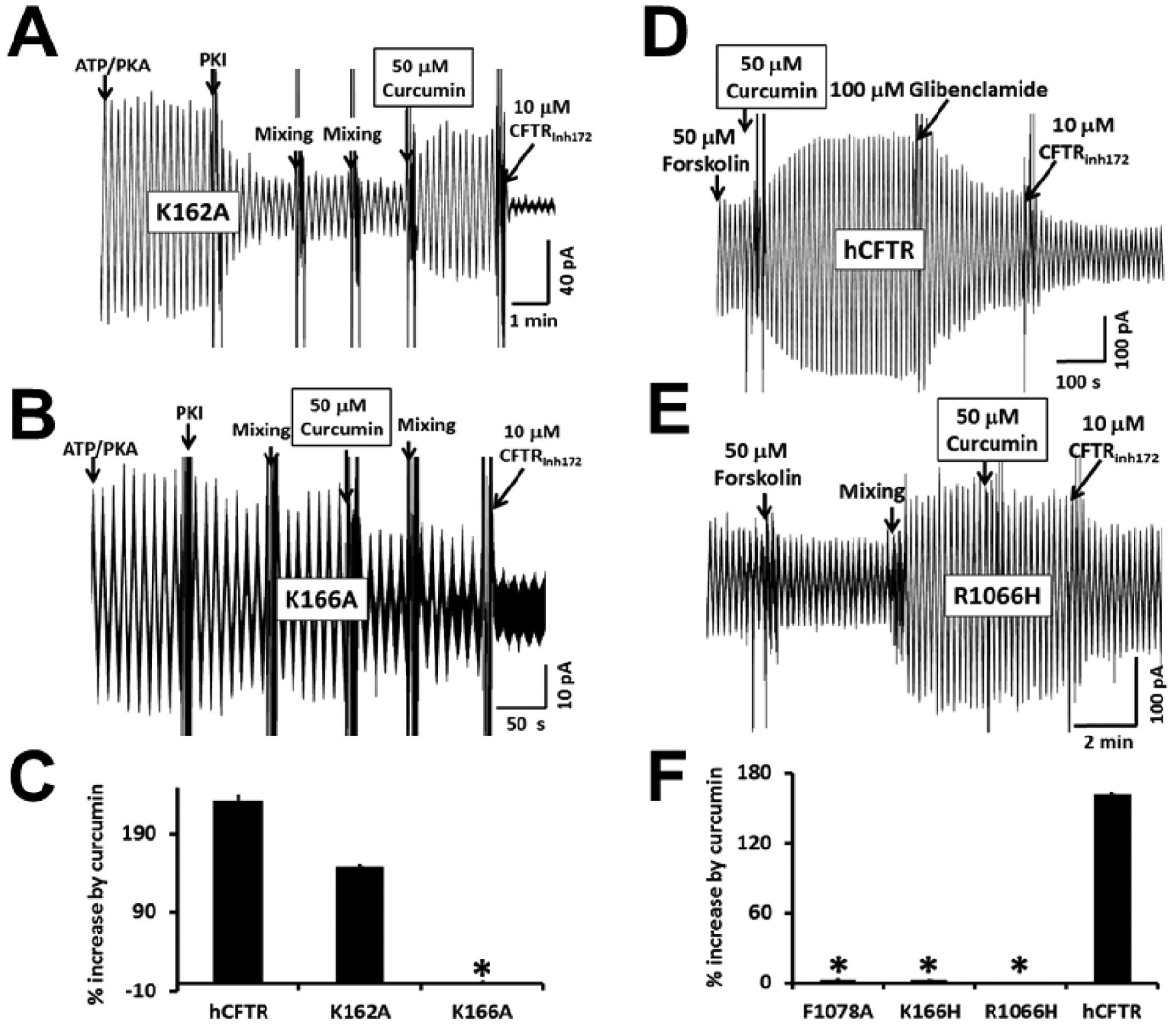


Figure 3. Effects of positively charged residues at the ICL1/ICL4 interface on curcumin potentiation. Inside-out macroscopic currents of (A) K162A and (B) K166A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA and followed by PKI. $CFTR_{inh172}$ (10 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. (C) Relative curcumin-induced potentiation of currents across inside-out membrane patches (n = 3; *P < 0.001, from an unpaired Student's t test). Whole-cell macroscopic currents of (D) WT hCFTR and (E) R1066H evoked by a ramp from 20 to −20 mV. The channel was activated with forskolin (50 μM). $CFTR_{inh172}$ (10 μM) or glibenclamide (100 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. (F) Relative curcumin-induced whole-cell current potentiation (n = 3; *P < 0.001, from an unpaired Student's t test).

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 with a Student's t test.

RESULTS

Aromatic Residues at the ICL1/ICL4 Interface Are Sensitive to Curcumin. Because two aromatic rings of curcumin can form a $\pi-\pi$ interaction with an aromatic residue in CFTR,[31] I first investigated Y161 from ICL1 and F1078 from ICL4, which are well paired in the inward- and outward-facing TMDs and thus facilitate curcumin binding (Figure 1A,B). Figure 1D indicates that these two aromatic residues are highly conserved across CFTR species, including hCFTR and mCFTR. Therefore, it is fitting to ask if they are sensitive to curcumin. Panels A and B of Figure 2 show that both hCFTR (Figure 2A) and mCFTR (Figure 2B) were activated by 1.5 mM ATP and 24 units/mL PKA, and their activity was further potentiated by 50 μM curcumin after PKI pretreatment. Their total currents were inhibited by $CFTR_{inh172}$ (10−20 μM), confirming that the increase in currents was due to the curcumin potentiation of CFTR activity instead of the deterioration of the patch seal. However, the curcumin potentiation of hCFTR activity was more dramatic (260%) than that of mCFTR activity (140%). Because the difference in two interface residues (R950 and T775) causes mCFTR to be insensitive to Fe^{3+},[28] it is reasonable that curcumin may potentiate mCFTR only in an Fe^{3+}-independent manner.[29] It is interesting that Y161A from ICL1 or F1078A from ICL4 was not potentiated by curcumin, although Fe^{3+} can still bind to the R−ICL3 interface (Figure 2C,D). In contrast, an alanine substitution of other aromatic residues in hCFTR failed to completely suppress curcumin potentiation, for example, F1026A of TM10, W1098A and F1107A of TM11, F1068A of ICL4, F319A of TM5, W361A of the C helix of TM6, and F490A and W496A of NBD1 (Figure 2D). Thus, both Y161 and F1078 may play a critical role in Fe^{3+}-dependent and Fe^{3+}-

DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840

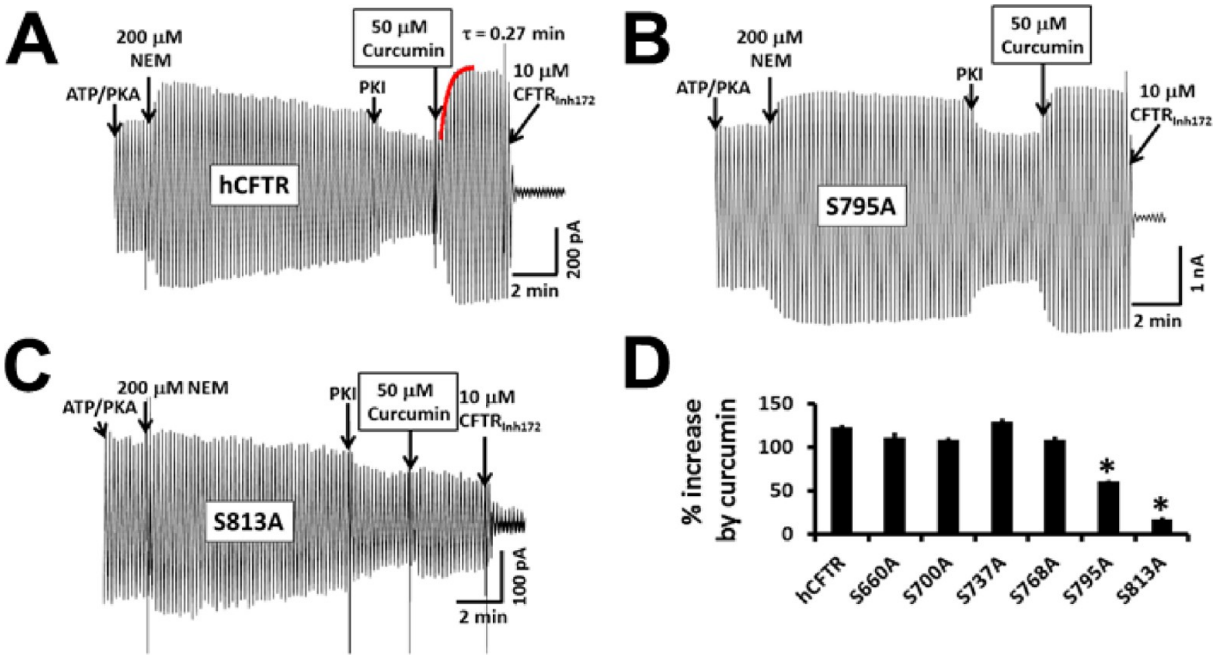


Figure 4. PKA site-dependent curcumin potentiation in the presence of NBD1−NBD2 dimerization. Inside-out macroscopic currents of (A) hCFTR, (B) S795A, and (C) S813A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA and pretreated with PKI. $CFTR_{inh172}$ (10 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. The red line is the fit of curcumin potentiation to a single-exponential function. Time constant τ = 0.24 ± 0.07 min (n = 5). (D) Relative curcumin-induced current increases of CFTR mutants after NEM modification (n = 3−5; *P < 0.001, from an unpaired Student's t test).

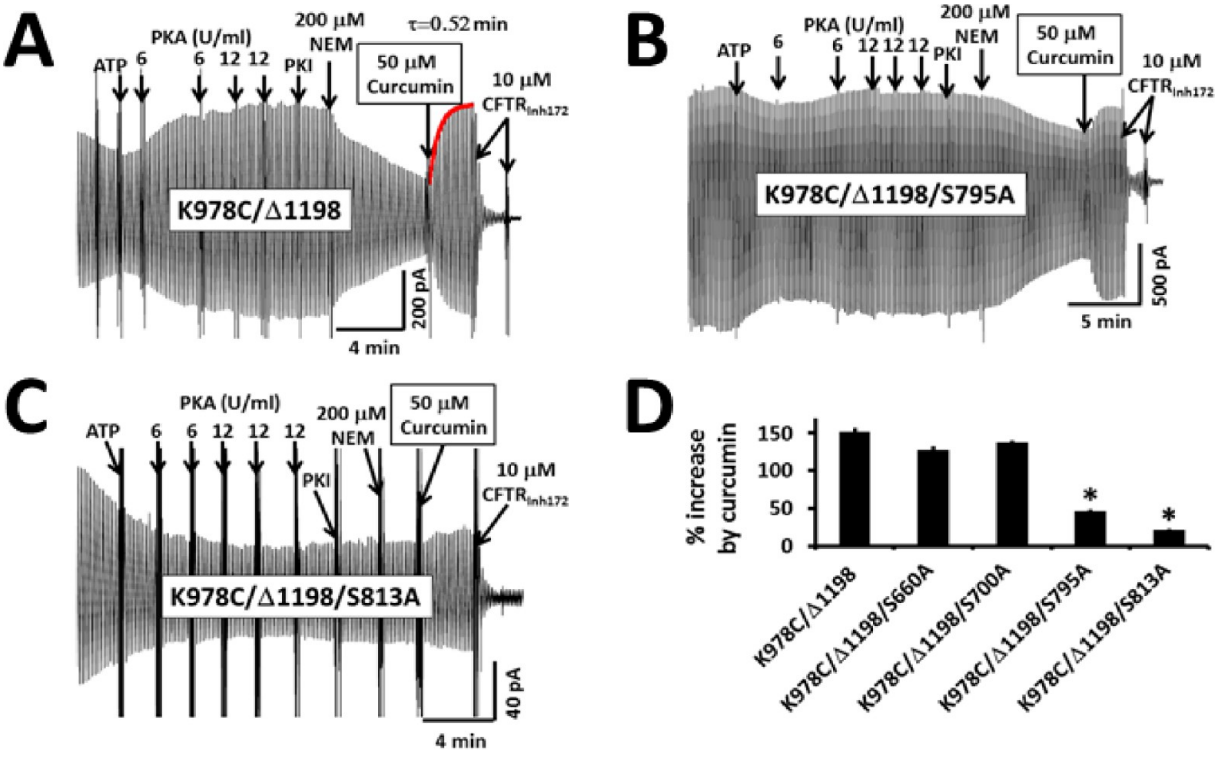


Figure 5. PKA site-dependent curcumin potentiation in the absence of NBD1−NBD2 dimerization. Inside-out macroscopic currents of (A) K978C/Δ1198, (B) K978C/Δ1198/S795A, and (C) K978C/Δ1198/S813A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 12−48 units/mL PKA and followed by PKI. $CFTR_{inh172}$ (10 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. The red line is the fit of curcumin potentiation to a single-exponential function. Time constant τ = 0.61 ± 0.19 min (n = 3). (D) Relative curcumin-induced current increases of CFTR mutants after NEM modification (n = 3−5; *P < 0.001, from an unpaired Student's t test).

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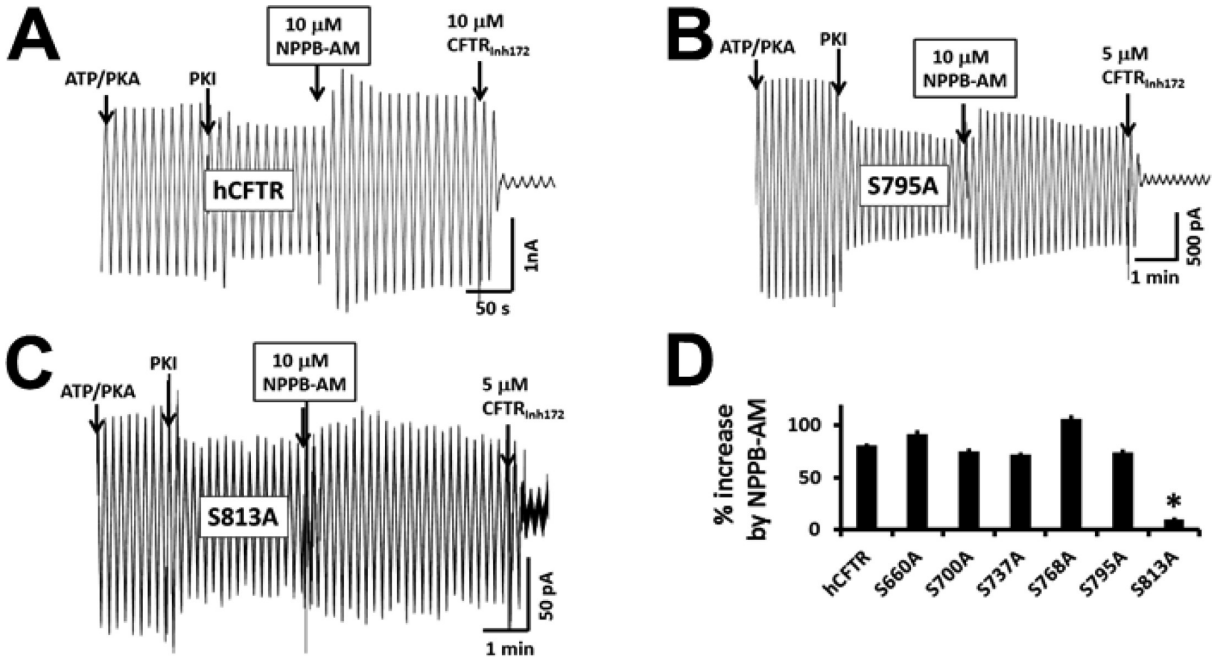

Figure 6. PKA site-dependent NPPB-AM potentiation. Inside-out macroscopic currents of (A) hCFTR, (B) S795A, and (C) S813A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA after pretreatment of EDTA (2.4 mM). Further phosphorylation was blocked by PKI. $CFTR_{inh172}$ (5−10 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. (D) Relative NPPB-AM-induced current increases of CFTR mutants after pretreatment of 2.4 mM EDTA (n = 3−5; *P < 0.001, from an unpaired Student's t test).

independent curcumin potentiation. Western blot analyses further indicated that Y161A had regular protein expression but the F1078A mutation reduced the level of protein expression (Figure 2E,F).

Positively Charged Residues at the ICL1/ICL4 Interface Are Sensitive to Curcumin. Because two aromatic rings of curcumin can also form a cation−π interaction with a positively charged residue in CFTR,[31] I further investigated several positively charged residues at ICL1 or ICL4. Figure 3A shows that K162A, mutated next to curcumin-sensitive Y161, was still activated by 1.5 mM ATP and 24 units/mL PKA. The subsequent application of curcumin also potentiated the channel current after the channel was pretreated with PKI. In contrast, K166A was not sensitive to curcumin (Figure 3B,C). Because both K166 from ICL1 and R1066 from ICL4 are highly conserved across species and close to each other in the inward- and outward-facing TMDs (Figure 1B,D), it is fitting to ask whether R1066 is also sensitive to curcumin. On the other hand, the R1066H mutation has been reported to decrease the extent of processing but to retain the normal channel function.[37,38] Therefore, the whole-cell current was measured to maximize the curcumin effect. Figure 3D indicates that curcumin (50 μM) significantly increased WT hCFTR activity by 160% after the channel was activated by 50 μM forskolin and the increase was blocked by 100 μM glibenclamide and 10 μM $CFTR_{inh172}$. In sharp contrast, R1066H (Figure 3E) or K166H (Figure 3F) activity was evoked by forskolin but not potentiated by 50 μM curcumin, while the Fe^{3+}-binding interface was not changed. Thus, both K166 and R1066 may also contribute to Fe^{3+}-dependent and Fe^{3+}-independent curcumin potentiation. In addition, the whole-cell current of F1078A was also not responsive to curcumin once the mutant was activated with forskolin (Figure 3F). Thus, both inside-out and whole-cell configurations can be used to define the curcumin sensitivity.

Curcumin Potentiation Is PKA Site-Dependent. CFTR has six critical PKA sites with the highly conserved motif [R(K)R(K)xSx] and distance between them.[39,40] They include stimulatory S660, S700, S795, or S813 and inhibitory S737 and S768 (Figure 1C).[41,42] Because the curcumin potentiation of hCFTR is PKA-dependent,[19] it is fitting to identify which PKA site is responsible for it. Figure 4A shows that hCFTR was activated by 1.5 mM ATP and 24 units/mL PKA and markedly potentiated by curcumin even after NEM modification to remove the Fe^{3+}-binding site.[28,43] Similar results were also seen with S660A, S700A, S737A, and S768A (Figure 4D). In sharp contrast, an S795A (Figure 4B) or S813A (Figure 4C) mutation significantly suppressed curcumin potentiation after NEM modification (Figure 4B−D). It is noteworthy that the NEM effect of S813A was also weakened (Figure 4C). Thus, PKA site S795 or S813 may contribute to Fe^{3+}-independent curcumin potentiation, and S813 may also be responsible for NEM potentiation.

PKA Site-Dependent Curcumin Potentiation Needs Not NBD1−NBD2 Dimerization. Previous studies demonstrated that the PKA-dependent curcumin potentiation of CFTR activity needs not ATP-binding-induced NBD1−NBD2 dimerization.[19,22] Therefore, to further measure if the stimulatory S795 or S813 site is still sensitive to curcumin in the absence of NBD1−NBD2 dimerization, I examined the curcumin effect by using K978C/Δ1198 CFTR as a control. Figure 5A demonstrated that K978C/Δ1198 CFTR was constitutively active and maximally stimulated not by ATP but with PKA (12 units/mL), suggesting that binding of the phosphorylated R domain to cytoplasmic domains other than NBD1 and ICL3 can directly stimulate channel activity.[19,22,26,28,29] In this case, the maximal channel activity of

DOI: 10.1021/acs.biochem.5b00219
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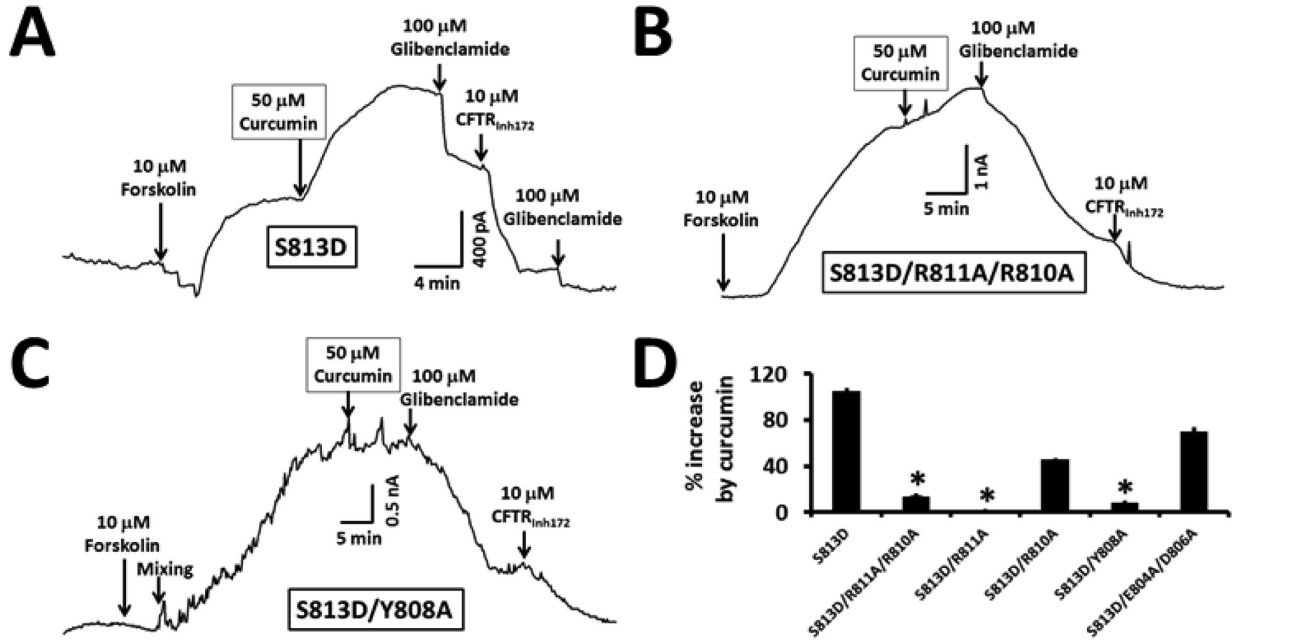

Figure 7. Effects of mutations at PKA site S813 on curcumin potentiation. Whole-cell currents of (A) S813D, (B) S813D/R810A/R811A, and (C) S813D/Y808A transfected in a HEK-293T cell at a holding potential of 40 mV. The channel was activated with 10 μM forskolin. $CFTR_{inh172}$ (10 μM) or glibenclamide (100−200 μM) was used to block the total CFTR-mediated current. Arrows indicate the time at which each reagent was added. (D) Relative curcumin-induced current potentiations (n = 3; *P < 0.001, from an unpaired Student's t test).

the mutant cannot be further potentiated by curcumin but can be decreased by modification of K978C.[22,29] Therefore, after the channel was pretreated with PKI and the channel current was inhibited by NEM modification of K978C, curcumin (50 μM) was found to increase the mutant activity by 152% and the increase was blocked by 10 μM $CFTR_{inh172}$. It is noteworthy that removal of NBD2 significantly slowed curcumin potentiation, although both hCFTR and K978C/Δ1198 activity could be potentiated by curcumin in a single-exponential growth manner (time constant τ, from ~0.24 to ~0.61 min) (Figures 4A and 5A). Similar results were observed with the insertion of S660A or S700A (Figure 5D). In contrast, a reduced dose of PKA (6 units/mL) was enough to stimulate the constitutively active K978C/Δ1198/S795A CFTR (Figure 5B). After the mutant was pretreated with PKI and the mutant current was inhibited by NEM, curcumin had a weak effect on the channel current. Furthermore, constitutively active K978C/Δ1198/S813A CFTR had no response to not only ATP, PKA, PKI, and NEM but also curcumin (Figure 5C). Because NEM pretreatment can also remove Fe^{3+} at the ICL3−R interface, it is reasonable that the inhibition of K978C/Δ1198 activity by NEM was enhanced by the C832A mutation but the subsequent curcumin potentiation was similar to that of NEM-modified hCFTR (Figures 4A and 5A).[29] Thus, the Fe^{3+}-independent curcumin sensitivity of PKA site S795 or S813 was independent of NBD1−NBD2 dimerization.

PKA Site S813 Is Sensitive to the NPPB-AM. Because the NPPB-AM potentiation of hCFTR is NBD2- and Fe^{3+}-independent,[29,44] and NPPB-AM has not the β-diketone moiety but two linked aromatic rings that can also form a putative $\pi-\pi$ or cation$-\pi$ interaction with the ICL1/ICL4 interface, it is fitting to ask whether NPPB-AM shares the same binding site with curcumin. Figure 6A shows that hCFTR was activated by 1.5 mM ATP and 24 units/mL PKA and potentiated by NPPB-AM after EDTA (2.4 mM) pretreatment to remove Fe^{3+},[29] and PKI pretreatment to prevent further phosphorylation. Similar results were also observed with S660A, S700A, S737A, S768A, and S795A (Figure 6B,D). In sharp contrast, the S813A mutation was not responsive to NPPB-AM after EDTA (2.4 mM) pretreatment (Figure 6C,D). Accordingly, PKA site S813 may be responsible for the Fe^{3+}-independent potentiation of CFTR by both NPPB-AM and curcumin.

The Basic and Aromatic Residues of PKA Site S813 Contribute to Curcumin Potentiation. Because the S813A mutation completely suppressed curcumin or NPPB-AM potentiation, it is necessary to examine whether a negative charge introduced by the addition of a phosphoryl group upon S813 phosphorylation is responsible for curcumin potentiation. Here, I used S813D to mimic phosphorylation of S813 to test this hypothesis. Figure 7A indicates that the whole-cell current of S813D was stimulated by forskolin (10 μM), dramatically potentiated by external curcumin (50 μM), and finally inhibited by glibenclamide (200 μM) and $CFTR_{inh172}$ (10 μM). Figure 7D indicates that the insertion of E804A/D806A failed to change the curcumin sensitivity of S813D CFTR. However, the whole-cell current of S813D/R810A/R811A was only slightly increased by curcumin at steady activation by forskolin (10 μM) (Figure 7B). Further study demonstrated that R811A may be mainly responsible for curcumin insensitivity (Figure 7D). A similar result was also observed with S813D/Y808A CFTR (Figure 7C). Therefore, not the negative charge but R811 and Y808 of the PKA site S813 may contribute to curcumin potentiation upon S813 phosphorylation. On the other hand, because endogenous Fe^{3+} can still bind to the ICL3−R interface of the mutants mentioned above, R811 and Y808 at PKA site S813 may finally contribute to Fe^{3+}-independent and Fe^{3+}-dependent curcumin potentiation.

ICL1−R Disulfide Cross-Linking Promotes PKA-Dependent Channel Opening. Because the curcumin sensitivity of the R domain started from critical stimulatory C-terminal PKA site S795 of the R domain and the ICL1/ICL4

DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840

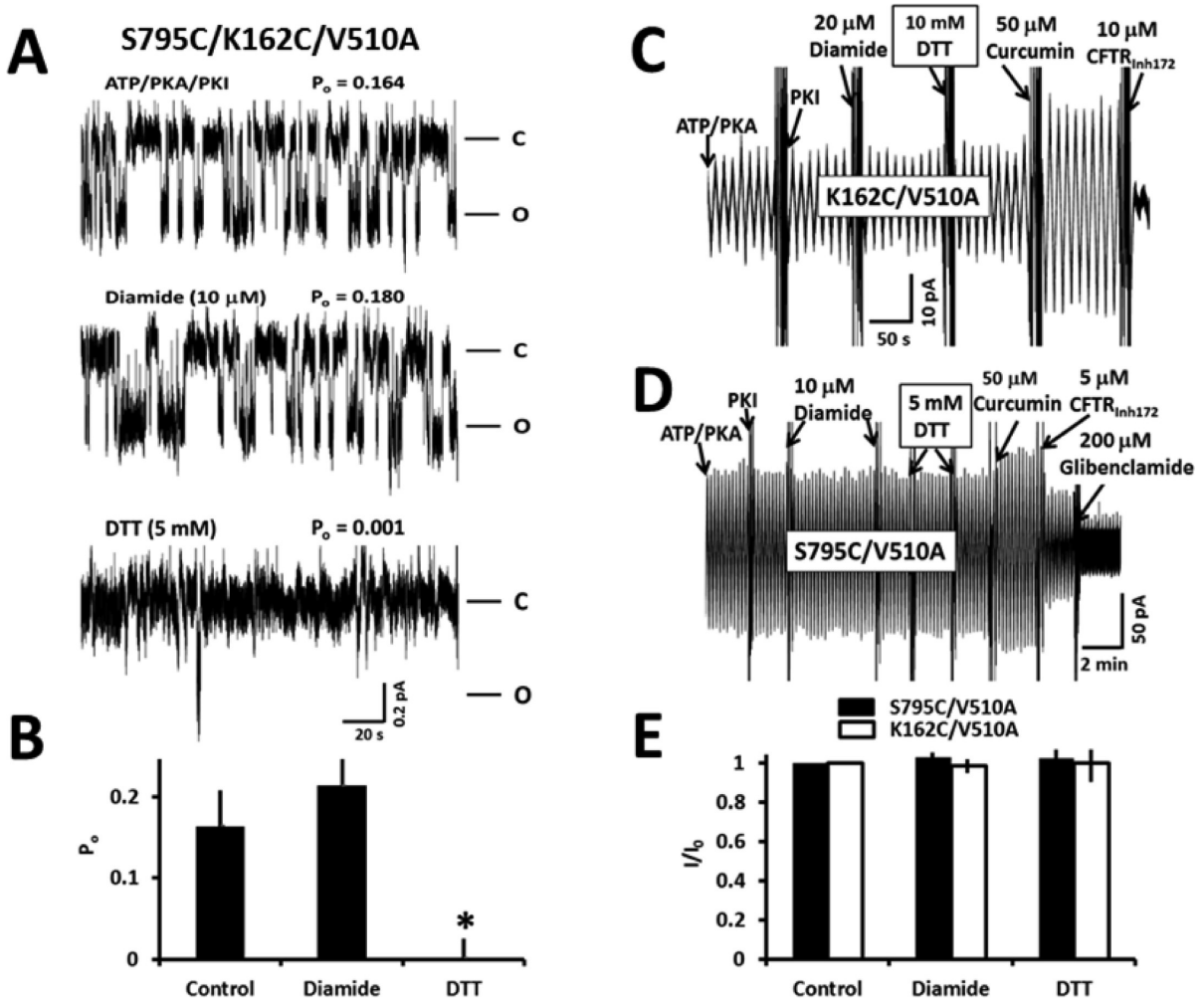

Figure 8. Effects of spontaneous disulfide cross-linking of K162C to S795C on single-channel opening. (A) Representative inside-out single-channel currents of S795C/K162C/V510A across an excised HEK-293T micropatch. The holding potential was −60 mV. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA, followed by PKI. Diamide was used to induce formation of a disulfide bond, while DTT was employed to disrupt the disulfide bond. (B) Changes in the open probability of S795C/K162C/V510A (n = 3; *P < 0.001, from an unpaired Student's t test). Inside-out macroscopic currents of (C) K162C/V510A and (D) S795C/V510A across an excised HEK-293T patch. The channel was activated with 1.5 mM MgATP and 24 units/mL PKA and followed by PKI (1.4 μg/mL). CFTR$_{inh172}$ (5−10 μM) or glibenclamide (200 μM) was used to block the total CFTR-mediated current. (E) Relative changes in currents in response to diamide (10−20 μM) and DTT (5−10 mM) (n = 3; *P < 0.001, from an unpaired Student's t test).

interface is highly conserved in the inward- and outward-facing TMDs, it is fitting to ask if disulfide cross-linking of K162 of ICL1 to PKA site S795 promotes channel opening. Panels A and B of Figure 8 show that diamide (10 μM) only slightly increased the open probability (P_o) of S795C/K162C/V510A/Cys-free CFTR from 0.164 to 0.180 after the channel was activated by 1.5 mM ATP and 24 units/mL PKA and pretreated with PKI. However, subsequent DTT (5 mM) dramatically reduced the open probability from 0.180 to 0.001 but did not alter single-channel current amplitude. The decrease in P_o primarily resulted from an increase in the interburst interval. In contrast, K162C/V510A/Cys-free or S795C/V510A/Cys-free CFTR was irresponsive to diamide or DTT but still potentiated by curcumin after the channel was activated by ATP and PKA and pretreated with PKI (Figure 8C−E). Therefore, a spontaneous disulfide cross-linking of K162C to S795C may be formed to maintain channel opening. Like S795A or K978C/Δ1198/S795A CFTR, S795C/V510A/Cys-free CFTR was weakly sensitive to curcumin (Figure 8D). In addition, because Fe^{3+}-sensitive C832 is mutated to serine in Cys-free CFTR, the stimulatory disulfide cross-linking between the R domain and ICL1 may be Fe^{3+}-independent.

Fe^{3+}-Independent Channel Opening of H775A and H950D/S768D CFTR Is Enhanced by Curcumin. Because the stimulatory disulfide cross-linking of the R domain to ICL1 described above was based on the Cys-free construct that removes the Fe^{3+}-binding interface by the C832S mutation,[9] it is fitting to ask whether the Fe^{3+}-independent curcumin potentiation of CFTR activity also promotes channel opening similarly. To test this hypothesis, I investigated the effect of curcumin on single-channel properties of two CFTR mutants. The H775A mutation can directly disrupt the Fe^{3+} bridge between the R domain and ICL3, while the H950D/S768D mutation can break up the H-bond between the imidazole group of H950 and the -OH group of S768 and the Fe^{3+} bridge between the R domain and ICL3 upon EDTA pretreatment so that C-terminal PKA site S795 or S813 of the R domain can be released from ICL3 to bind to the ICL1/ICL4 interface.[26,28] Figure 9 shows that after the channel was activated with 1.5 mM ATP and 24 units/mL PKA and pretreated with PKI and 5

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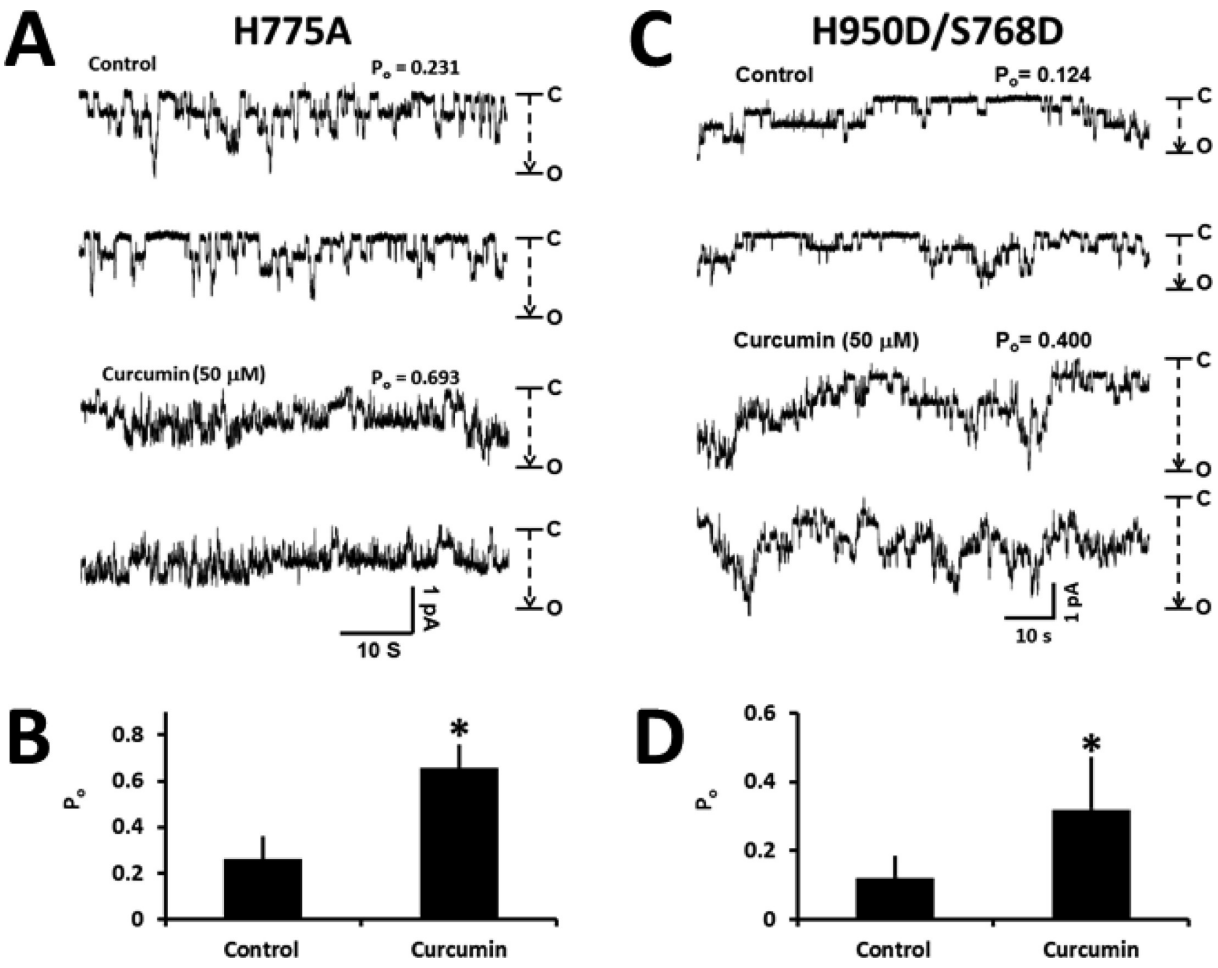

Figure 9. Curcumin stimulates channel opening of H775A CFTR and H950D/S768D CFTR. Representative inside-out unitary currents of (A) H775A and (C) H950D/S768D across an excised HEK-293T micropatch in the absence and presence of 50 μM curcumin. The holding potential was −60 mV. The channel was activated by 1.5 mM ATP and 24 units/mL PKA and pretreated with PKI and 5 mM EDTA. Effect of curcumin (50 μM) on single-channel open probabilities (P_o) of (B) H775A and (D) H950D/S768D (assuming the channel number per patch is the maximal number of simultaneous openings after curcumin addition) (n = 3; *P < 0.05, from an unpaired Student's t test).

mM EDTA, 50 μM curcumin significantly increased the channel open probabilities (P_o) of H775A and H950D/S768D by ~2.5-fold primarily by decreasing the interburst interval but did not alter the single-channel current amplitude. Taken together, these results strongly support the hypothesis that curcumin stabilizes the ICL1/ICL4−R interaction that may promote channel opening

DISCUSSION

R domain phosphorylation is required for CFTR activation and PKA-dependent curcumin potentiation. Elucidation of its activation or potentiation pathway is very important to the design of rational drugs to rescue CF-causing mutants with an ATP-dependent gating defect. My previous study demonstrated that disruption of inhibitory R−ICL3 interactions is required for CFTR activation and potentiation.[26,28,29,33] This study further proposes that the interactions between the highly conserved ICL1/ICL4 interface and the C-terminus of the R domain may also be necessary for the PKA-dependent channel activation and potentiation of CFTR. Therefore, this study may open a door for future medicinal chemistry optimization of CFTR potentiators and experimental therapeutics of patients with cystic fibrosis.

ICL1/ICL4−R Interactions Promote PKA-Dependent CFTR Activation. CFTR has six critical PKA sites with the highly conserved motif [R(K)R(K)xSx] and distance between them.[39,40] Although none is specifically responsible for PKA-dependent CFTR activation, S813 is the strongest.[39,41,45−48] Phosphorylation introduces negative charges into the R domain and thus decreases the α-helical propensity of segments 648−670 and 801−818,[16,49] changes its size, and increases its flexibility or mobility.[50−52] Thus, although the R domain with more hydrophilic and charged residues lacks a stably folded globular structure and thus is disordered,[16,50−52] once the PKA-phosphorylated R domain is released from NBD1 and ICL3, these highly conserved and repeat PKA sites may function as possible intramolecular recognition elements to interact with the ICL1/ICL4 interface so that the distance between TMD1 and TMD2 can be gating-regulatory (Figure 10). Putative strong cation−π interactions of the highly conserved positively charged R/K of the PKA sites with highly conserved aromatic amino acids Y161 and F1078 at the ICL1/ICL4 interface may glue ICL1/ICL4 and the R domain together (Figures 1 and 10D). Accordingly, except PKA site S660 that may be inaccessible to the ICL1/ICL4 interface and PKA site S700 that may form a stimulatory interaction with the N-terminal cytoplasmic tail of CFTR,[53] other PKA sites such as S737

DOI: 10.1021/acs.biochem.5b00219
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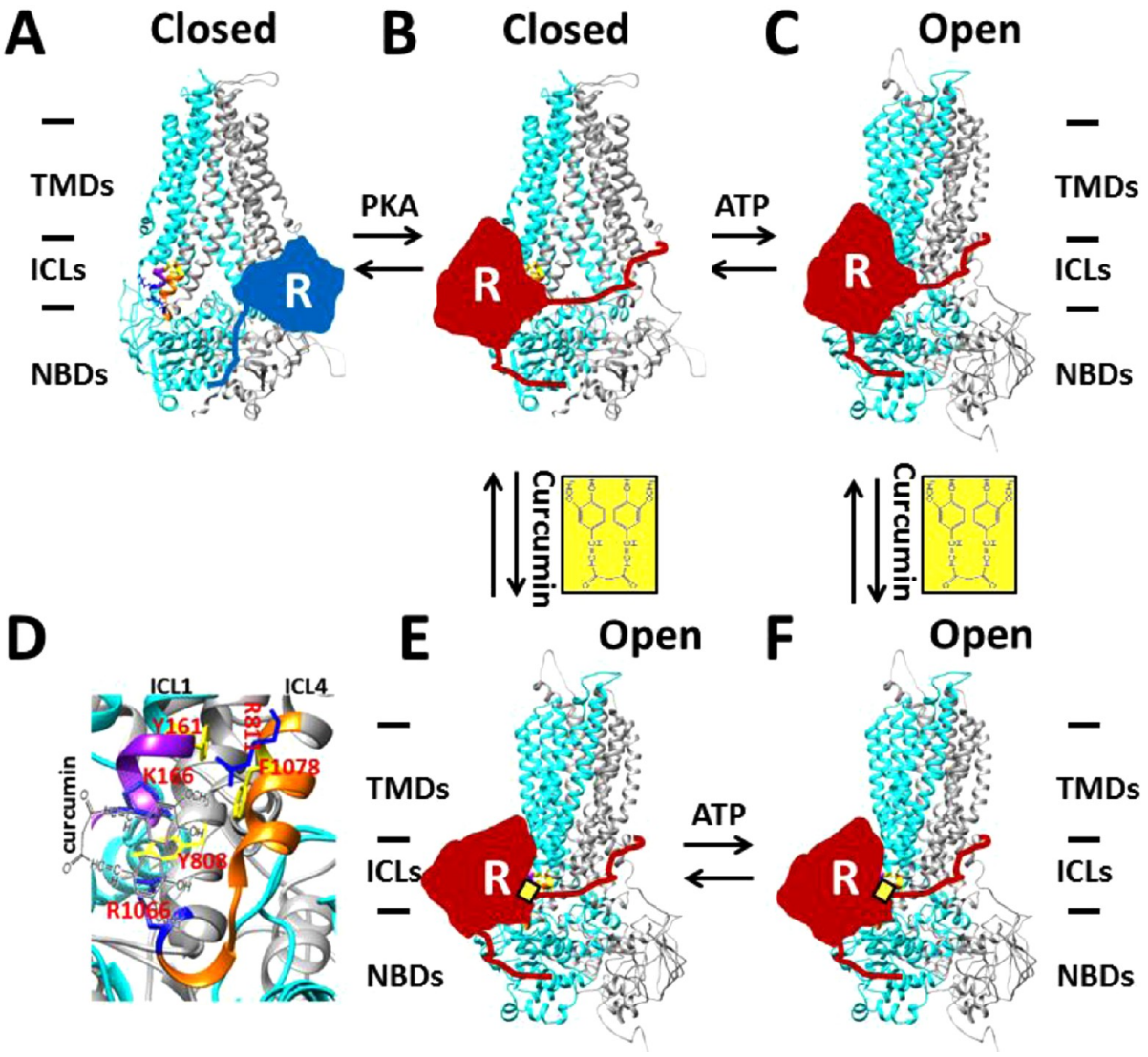

Figure 10. Tentative curcumin potentiation mechanisms. R domain phosphorylation by PKA releases itself from NBD1 and ICL3 (A and B) and thus promotes ATP-binding-induced NBD1−NBD2 dimerization (ATP not shown) (C), which opens the channel. However, curcumin can directly stabilize putative cation−π interactions of PKA site S813 with the ICL1/ICL4 interface, which pulls ICLs together for channel opening even without NBD2 (D and E). (D) R811 (blue) is sandwiched by Y161 (yellow) and F1078 (yellow) (Y161/**R811**/F1078), while K166 (blue) and R1066 (blue) are clamped by Y808 (yellow) and two aromatic rings of curcumin (gray) (ring 1/**K166**/Y808/**R1066**/ring 2). Taken together, ATP-binding-induced NBD1−NBD2 dimerization (ATP not shown) and curcumin-enhanced ICL1/ICL4−R interactions promote channel opening (F).

(EPL**ERR**LS$_{737}$), S768 (lqar**RRQ**S$_{768}$), S795 (taST**RK**vS$_{795}$), and S813 (**DIYSRR**LS$_{813}$) may compete for binding to the ICL1/ICL4 interface to tune the distance between the ICL1/ICL4 and ICL2/ICL3 interfaces. However, only the S813 site may most dramatically pull ICLs together once bound to the ICL1/ICL4 interface and thus most activate the CFTR channel without requiring NBD1−NBD2 dimerization (Figures 1C and 10). This proposal, in agreement with Medusa-based discrete molecular dynamics (DMD) simulations of the possible phosphorylation-induced structural changes of the R domain[40] and the stimulatory NBD2-independent electrostatic attraction between E267 from ICL2 and K1060 from ICL4,[54] is supported by the observations that the S813A mutation completely suppressed the stimulation of K978C/Δ1198 CFTR by PKA (Figure 5C) and that the insertion of R811A/R810A or Y808A clearly slowed S813D activation by forskolin (Figure 7A−C). The S795 site may be the second one to open the channel in this way (Figures 4B and 5B). In contrast, the binding of S768 and S737 sites to the ICL1/ICL4 interface may decrease a binding probability of the stimulatory S795 or S813 site at the ICL1/ICL4 interface and thus inhibit channel opening. Therefore, the ICL1/ICL4 interface may be a critical gating-regulatory active recognition site for some PKA sites of the R domain.

ICL1/ICL4−R Interactions Are Necessary for the Fe^{3+}-Dependent and Fe^{3+}-Independent Curcumin Potentiation of hCFTR Activity. Curcumin is a highly pleitropic molecule with three important functionalities such as a hydrophobic aromatic *o*-methoxy phenolic group, a flexible α,β-unsaturated β-diketo linker, and keto−enol tautomerism. Molecular docking studies have suggested that curcumin can directly bind by multiple forces such as an H-bond, a $\pi-\pi$ interaction, and a cation−π interaction to many proteins and enzymes, including protein kinases.[31] However, further X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy studies are necessary to identify such binding

DOI: 10.1021/acs.biochem.5b00219
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interactions. My previous investigation indicated that sufficient Fe^{3+} can inhibit curcumin or NPPB-AM potentiation by binding to the ICL3−R interface.[29] Because this study further demonstrated that aromatic and positively charged residues at stimulatory PKA site S813 of the R domain and the highly conserved ICL1/ICL4 interface were deeply involved in Fe^{3+}-dependent and Fe^{3+}-independent curcumin potentiation (Figures 2, 3, and 7), PKA site S813 was sensitive to curcumin and NPPB-AM that both have two linked aromatic rings (Figures 4C and 6C), and disulfide cross-linking of K162C of ICL1 to C-terminal S795C of the R domain was enough to stimulate channel opening as curcumin did (Figures 8 and 9), it is likely that the highly charged and conserved K166 of ICL1 and R1066 of ICL4 may form cation−π interactions with two aromatic rings of curcumin and Y808 from the S813 PKA site, which enhance a putative cation−π interaction of R811 with Y161 and F1078 for channel opening (Figure 10D). This proposal was supported by the observations that the curcumin sensitivity of PKA site S813 was independent of NBD1−NBD2 dimerization (Figure 5C) and that NPPB or NPPB-AM with two linked aromatic rings can also potentiate CFTR activity while APB (2-amino-4-phenylbutyric acid, a half-curcumin-like molecule) with one aromatic ring cannot.[44] Because of Fe^{3+}-independent NPPB-AM or curcumin potentiation and Fe^{3+}-dependent curcumin potentiation,[29] it is reasonable that pretreatment of NPPB-AM cannot suppress curcumin potentiation but pretreatment of curcumin prevents NPPB-AM potentiation.[54] It is noteworthy that S813 should be phosphorylated to reduce the α-helical content or to expose R811 and Y808 to fit the ICL1/ICL4 interface well (Figure 10D). For S795A or K978C/Δ1198/S795A CFTR, modification of C832 with NEM may increase the control channel activity, and thus, subsequent curcumin potentiation may be apparently and relatively weakened (Figures 4B, 5B, and 8D).[43] In contrast, for S768A, S737A, S700A, or S660A with a WT or K978C/Δ1198 background, NEM modification of a cysteine other than C832 may decrease the control current so that curcumin potentiation was not significantly different from that of WT hCFTR or K978C/Δ1198 CFTR (Figures 4 and 5). Accordingly, PKA site S795 without a Y808-like aromatic ring may not facilitate curcumin's stabilizing the interaction between PKA site S795 and the ICL1/ICL4 interface. Consistent with this proposal, this site was insensitive to NPPB-AM after EDTA removed inhibitory Fe^{3+} at the ICL3−R interface (Figure 6B). In addition, because removal of NBD2 significantly slowed curcumin potentiation (Figures 4 and 5) and the same ICL1/ICL4−NBD1 and ICL2/ICL3−NBD2 swapping interactions, shared by both inward- and outward-facing TMDs, facilitate gating regulation by ATP,[2−4,6] the ICL1/ICL4−R interactions may also be enhanced by ATP-binding-induced NBD1−NBD2 dimerization (Figure 10F).

Implications for Cystic Fibrosis Treatment. Loss of function mutations in CFTR cause cystic fibrosis (CF), the most common monogenic inherited lethal disorder among Caucasians. Mechanistic insights into CFTR gating may aid drug development of novel CFTR potentiators that restore conductance defects of mutant CFTR.

The overwhelming clinical success of recently Food and Drug Administration-approved CF drug Kalydeco (VX-770) highlights the validity of the CFTR potentiator in treating CF at its basic defect.[55] A more complete picture of the gating mechanism of CFTR may lead to the discovery of additional drugs that potentiate other CFTR mutants with comparable clinical efficacy. Several powerful CFTR potentiators have been identified by high-throughput screening. Elucidation of their potentiation pathways is very important for future medicinal chemistry optimization. Because both curcumin and VX-770 promote CFTR activation in an ATP-independent but PKA-dependent manner,[19,56] and pretreatment of VX-770 prohibits curcumin potentiation,[54] they may share a similar potentiation pathway. My previous study demonstrated that removal of endogenous Fe^{3+} to release the R domain from ICL3 is necessary for PKA-dependent potentiation.[29] In this study, the stimulatory ICL1/ICL4−R interactions may further be necessary to bypass the need for ATP to potentiate certain defective CFTR alleles responsible for CF. Therefore, these abnormal and complex PKA-dependent potentiation mechanisms may help improve our knowledge of CFTR gating and optimize CFTR potentiators and promote the development of a cost-effective single-drug therapeutic strategy for patients with CF.

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Funding

American Heart Association (AHA) Grant 10SDG4120011 (to G.W.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

I thank Drs. Jonathan Widdicombe, Jie Zheng, David Clarke, David Gadsby, Dayue Darrel Duan, Wei Wang, Kevin Kirk, Ge Li, and Yohei Norimatsu for their support.

ABBREVIATIONS

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; hCFTR, human CFTR; mCFTR, mouse CFTR; ABC, ATP-binding cassette; R, regulatory; TM, transmembrane; TMD, TM domain; NBD, nucleotide-binding domain; PKA, protein kinase A; PKI, protein kinase inhibitory peptide; EM, electron microscopy; NMR, nuclear magnetic resonance; DMD, discrete molecular dynamics; HEK, human embryonic kidney; ICL, intracellular loop; NPPB-AM, 5-nitro-2-(3-phenylpropylamino)benzamide; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate; APB, 2-amino-4-phenylbutyric acid.

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DOI: 10.1021/acs.biochem.5b00219
Biochemistry 2015, 54, 2828−2840