Regulation of Activation and Processing of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by a Complex Electrostatic Interaction between the Regulatory Domain and Cytoplasmic Loop 3*

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*Running title: Asymmetric electrostatic regulation of CFTR

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Background: NEG2 regulates CFTR gating but the mechanism is unknown.

Results: A putative NEG2-CL3 electrostatic attraction, possibly weakened by R764/R766 of the R domain, prohibited CFTR activation. A charge exchange between NEG2 and CL3 caused misprocessing.

Conclusion: Electrostatic regulation of CFTR activation and processing may be asymmetric at the CL3-R interface.

Significance: The CL3-R interface is optimally designed for multiple regulations of CFTR functions.

SUMMARY

NEG2, a short C-terminal segment (817-838) of the unique regulatory (R) domain of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, has been reported to regulate CFTR gating in response to cAMP-dependent R domain phosphorylation. The underlying mechanism, however, is unclear. Here, K946 of cytoplasmic loop 3 (CL3) is proposed as counter-ion of D835, D836 or E838 of NEG2 to prevent the channel activation by PKA. R764 or R766 of the S768 phosphorylation site of the R domain is proposed to promote the channel activation possibly by weakening the putative CL3-NEG2 electrostatic attraction. First, not only D835A, D836A and E838A but also K946A reduced the PKA dependent CFTR activation. Second, both K946D and D835R/D836R/E838R mutants were activated by ATP and curcumin to a different extent. Third, R764A and R766A mutants enhanced the PKA-dependent activation. On the other hand, it is very exciting that D835R/D836R/E838R and K946D/H950D and H950R exhibited normal channel processing and activity while D835R/D836R/E838R/K946D/H950D was misprocessed and silent in response to forskolin. Further, D836R and E838R played a critical role in the asymmetric electrostatic regulation of CFTR processing and S768 phosphorylation may not be involved. Thus, a complex interfacial interaction among CL3, NEG2 and the S768 phosphorylation site may be responsible for the asymmetric electrostatic regulation of CFTR activation and processing.

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) chloride channel is a unique member of the ATP-binding cassette (ABC) family because it has a unique regulatory (R) domain flanked by two intracellular nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) (Fig. 1A) (1, 2). Both TMDs consist of six membrane-spanning helices which are extended to the...
intracellular side to form four long cytoplasmic loops (CLs) (3). These CLs interact with not only NBD1 and NBD2 but also the R domain (3-10). The pore formed by TMD1 and TMD2 is gated by binding/hydrolysis of ATP at the interface of NBD1 and NBD2 and regulated by R domain phosphorylation/dephosphorylation (11, 12). Although CLs have been found to play a central role in channel gating (6, 7, 9, 10, 13, 14), it is largely unknown how CLs couples ATP actions at NBDs to gating rearrangements of TMDs and how R domain phosphorylation by cAMP-dependent PKA mediates the coupling.

The R domain is a segment with 193 amino acids (645-838). It has multiple phosphorylation sites, most of which are stimulatory except S768 and S737 (9, 10, 12, 15-18). Recent functional studies revealed that NEG2 (1B), which is a small C-terminal segment (817-838) of the R domain and has a conserved helical region and a net charge of -9, plays a critical role in CFTR gating and trafficking in response to cAMP stimulation (19-20). Removal of NEG2 suppresses PKA-dependent activation and trafficking of CFTR (19-20). However, the underlying mechanism is still a mystery.

Recent functional and biochemical studies have shown that correct interdomain interactions of CFTR are required not only for its optimal activation but also for its optimal folding, biosynthesis and trafficking from endoplasmic reticulum to the plasma membrane (6, 8, 21-23). Our recent investigations uncovered that S768 inhibits the CFTR activity by interacting with CL3 no matter whether it is phosphorylated or not (9-10). In the non-phosphorylated state, the OH- group of S768 forms a putative H-bond with the imidazole group of H950 of CL3 (10). Once phosphorylated, S768 may enhance binding of Fe3+ to H950, H954, C832, D836 and H775 at the interface of the R domain and CL3 (9). Because C832 and D836 are residues of NEG2 and close to CL3 (19), we wondered if the interaction of NEG2 with CL3 modulates CFTR activation, and if the S768 phosphorylation site is involved (Fig. 1B).

Molecular Biology- Human wild type (WT) CFTR was subcloned into the pCDNA3 mammalian expression vector (Invitrogen). All the mutants were produced by using the QuickChange™ site-directed mutagenesis kit from Stratagene and confirmed by automated sequencing and Western blotting. A Cys-free construct without all 18 cysteines was provided by Dr. David Gadsby (Rockefeller University). ΔR-S660A-CFTR was provided by Michael Welsh (University of Iowa, Iowa City, IA).

Cell Culture and Transfection-Human embryonic kidney (HEK)-293T cells were transiently transfected with wild type (WT) or mutant CFTR cDNA using the Lipofectamine transfection kit (Invitrogen). Cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% fetal bovine serum and 1mM penicillin/streptomycin. For patch clamp recordings, all cells were grown on plastic coverslips and used 1-4 days post-seeding.

Patch Clamp Analysis- Some channels expressed in HEK-293T cells were recorded in inside-out configurations (Dagan 3900A, Dagan Corporation, Minneapolis MN) for intracellular application of reagents to the cytoplasmic face. CFTR currents were recorded in symmetrical solutions each containing (mM) 140 N-methyl-D-glutamine chloride, 3 MgCl2, 1 EGTA and 10 TES (pH7.3). The resulting resistance of borosilicate patch pipette was 3-4 MΩ in the bath solution. Macroscopic currents were evoked using a ramp protocol from +80 to -80 mV with a 10.75s time period and filtered at 200 Hz. Single-channel recordings at 60mV are filtered at 20Hz. 1.5 mM MgATP and 100 units/ml PKA were used to activate the CFTR channel. For those channels expressed in HEK-293T cells and recorded in whole-cell configurations, 1.5mM ATP was introduced in the electrode solution, and 50µM forskolin and 50µM curcumin were used to estimate the current density of some CFTR mutants. All the experiments were carried out at room temperature (22±1°C). Data were acquired and analyzed using pCLAMP8.1 software (Axon Instruments). Data are shown as mean ± SEM. A student’s t test was used to test statistical
significance. Curve fitting was made using Microcal Origin software.

Western Blot Analysis- Transfected HEK-293T cells expressing CFTR WT and mutants were washed in divalent-free PBS (Mediatech, Herndon, VA) and then solubilized in 100 μl SDS sample buffer for SDS/PAGE analysis. Each sample was running on a 4-15% SDS PAGE gel. Separated proteins were transferred onto PVDF membranes for 60 min at 24 V (Genie blotter, Research Products International Corp, IL). The membranes were blocked for 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 1:5000 and detected with goat anti-mouse Alexa Fluor 680-conjugated antibody (Molecular probes, Eugene, OR) at 1:100,000. Blots were extensively washed by a TBS buffer and then scanned to obtain fluorescent images with an Odyssey scanner (LI-COR).

RESULTS

Electrostatic expulsions between the R domain and CL3 reduce the PKA-dependent channel activation – To address if a putative electrostatic attraction between CL3 and NEG2 regulates CFTR gating, we first determined the PKA-dependent activity of several mutants at the CL3-NEG2 interface. Fig. 2A indicates that low concentration (6 units/ml) of PKA activated WT CFTR slowly and weakly. In contrast, mutation of K946 or D836 to alanine clearly accelerated the channel activation and reduced the PKA-dependence (Fig. 2B and 2C). Similar observations with D835A and E838A were summarized in Fig. 2D. The K_{1/2} for PKA activation reduced from 10 units/ml to 5 units/ml once K946 from the CL3, and D835, D836 and E838 from NEG2 were mutated to alanines (Fig. 2D). However, K951A failed to change the PKA-dependent channel activity (Fig. 2D). Thus, a putative electrostatic attraction between K946 and D835, D836, or E838 may weaken the PKA sensitivity of CFTR activation.

To further confirm the putative electrostatic attraction between CL3 and NEG2, we determined the effects of curcumin on the mutants at the CL3-NEG2 interface. In the presence of 1.5mM ATP, 50μM curcumin greatly potentiated the K946A mutant activity, which was further increased by PKA (Fig. 3A). If this effect is due to a disruption of the putative electrostatic interaction between CL3 and NEG2, K946D or D835R/D836R/E838R should exhibit the similar effect in the presence of ATP. To support this argument, ATP and curcumin partially increased the K946D activity and PKA (6 units/ml) completely activated this mutant (Fig. 3B). In contrast, ATP and curcumin dramatically activated the D835R/D836R/E838R mutant. However, addition of PKA decreased the channel activity (Fig. 3C). This decrease in the channel current may be due to the side effect of curcumin (24). It is interesting that ATP and curcumin only activated the K946D mutant by 40% but activated the D835R/D836R/E838R mutant completely (Fig. 3D). Thus, the regulation of CFTR activation by the putative electrostatic attraction between K946 and NEG2 may be limited and asymmetric. The triple arginine mutant of NEG2 may not only disrupt the putative electrostatic interaction with K946 but also weaken the Fe^{3+} binding at the CL3-R interface because D836 is one of important Fe^{3+} ligands (9). However, for K946D, H950 or H954 may still form the inhibitive Fe^{3+} bridge with the R domain. In order to support this argument, K946D/H950D was employed to weaken both the electrostatic attractions and the Fe^{3+} bridge between CL3 and the R domain. Fig. 3D demonstrates that ATP and curcumin completely activated the K946D/H950D mutant. This high activity may not be due to the H950D mutation because it cannot be activated by ATP and curcumin (10). Thus, the inhibitive Fe^{3+} bridge at the R-CL3 interface should be weakened when the electrostatic interactions at the same interface are investigated.

On the other hand, previous studies demonstrated that CFTR can be activated by ATP alone and PKA does not exert any effect on the channel activation when NEG2 is deleted (19). If this ATP-induced activation results from the disruption of the putative electrostatic attraction between CL3 and the C-terminal of NEG2, the similar effect should be observed. However, neither K946D nor D835R/D836R/E838R was fully activated by ATP alone (Fig. 3B and 3C).
The asymmetric effects of R-CL3 electrostatic attractions on the CFTR activity – To further investigate the ATP-dependent activity of the CFTR mutants at the R-CL3 interface, the whole-cell CFTR currents were recorded to maximize the ATP effect on the channel activity when 1.5mM ATP was introduced in the electrode solution. Even if the activity of the inside-out patch expressing a CFTR construct is very low in the presence of ATP, any ATP-induced effect should be observed in the whole-cell configuration. Fig. 4A indicates that when the whole-cell current was determined with D835R/D836R/E838R, the application of external forskolin to the bath solution increased the mutant current. It is interesting that external curcumin continued to potentiate the mutant activity. Finally, glibenclamide or CFTR inh172 completely suppressed the mutant current (Fig. 4A). A similar case was observed with H950R (Fig. 4B). Because the D835R/D836R/E838R mutant exhibited the lower current with forskolin than H950R or WT CFTR, a cell capacitance was measured with WT and D835R/D836R/E838R CFTR constructs before and after forskolin was applied to evaluate the contribution of trafficking (20). Table 1 demonstrates that the cell capacitances of both WT and D835R/D836R/E838R were stable upon activation by forskolin. Thus, the low forskolin-stimulated current of the latter may not be due to trafficking.

Although both D835R/D836R/E838R and H950R were activated by forskolin and curcumin greatly, the introduction of K946D and H950D to D835R/D836R/E838R prevented the channel activation by forskolin and curcumin (Fig. 4C and 4D). It is very attractive that the small basic conductance was clearly observed before the inhibition by glibenclamide (Fig. 4C). In contrast, D835R/D836R/E838R and H950R failed to exhibit the basic activity (Fig. 4A and B). Thus, D835, D836 and E838 may not be responsible for the channel activation by ATP. Other parts of NEG2 may interact with other domains or other parts in the R domain to remove the PKA-dependent channel activation.

Upon normalization of the channel current to the cell capacitance, the insertion of K946D/H950D to D835R/D836R/E838R dramatically reduced the CFTR current density (Fig. 4D). In contrast, D835R/D836R/E838R and K946D/H950D and H950R exhibited comparable current densities (Fig. 4D). Thus, the putative electrostatic attractions between CL3 and the R domain may not be exchangeable to suppress the channel activity. In other words, the NEG2-CL3 electrostatic effects on the channel activity may be asymmetric. The putative electrostatic attraction between K946/H950R and D835/D836/E838 failed to inhibit the channel activity but the putative electrostatic attraction of D835R/D836R/E838R with K946D/H950D greatly suppressed the channel activity.

The asymmetric effects of R-CL3 electrostatic attractions on CFTR processing – To further determine if the reduction in the current density of K946D/H950D/D835R/D836R/E838R originates from the decrease in the channel expression or the single channel conductance, we carried out western-blotting analysis. Fig. 5 shows that both WT and ΔR CFTR constructs had a very strong mature band C but a very weak immature band B, suggesting that the deletion of the R domain had no effect on channel processing. The similar cases were seen with D835R/D836R/E838R and H950R and K946D/H950D (Fig. 5). However, the insertion of K946D/H950D to D835R/D836R/E838R clearly reduced the fractional mature Band C by 50%. Thus, the asymmetric electrostatic regulation of the channel activity at the NEG2-CL3 interface may be due to the asymmetric electrostatic regulation of channel processing. On the other hand, the channel activity of D835R/D836R/E838R/K946D/H950D was still very low (Fig. 4D). Therefore, it is also possible that the putative electrostatic CL3-R attraction may also stop the channel opening (10).

The effects of S768 phosphorylation on the asymmetric NEG2-CL3 electrostatic regulation of the CFTR activity and processing – Although previous studies demonstrated that S768 phosphorylation had no electrostatic contribution to the channel gating, non-phosphorylated S768 may form a strong putative H-bond with H950D to affect the CL3-NEG2 electrostatic interaction.
Therefore, we prepared a control mutant S768D/K946D/H950D to exclude this putative H-bond. Fig. 4D indicates a high current density of S768D/K946D/H950D based on a whole-cell patch recording. The introduction of D835R to this mutant failed to change the current density. However, the insertion of D836R or E838R to S768D/K946D/H950D dramatically reduced the CFTR current density (Fig. 4D). Fig. 5 further demonstrates that the fractions of the mature Band C of S768D/K946D/H950D/D836R and S768D/K946D/H950D/E838R were also significantly decreased by 45%. Thus, the reduction of their current densities may result from the decrease in the channel processing and opening (10). Taken together, the asymmetric electrostatic regulation of CFTR activation and processing was not due to the putative H-bond.

On the other hand, because S768D mimics S768 phosphorylation and H950R/S768D also exhibited the high channel activity (10), S768 phosphorylation failed to change the asymmetric electrostatic regulation of CFTR activation and processing.

The effects of R764A and R766A on the PKA-dependent channel activation – Because both S768 and D836 are close to H950 of CL3, it is fitting to ask if R764, R765 and R766 form a salt bridge with the N-terminal of NEG2 to cause the asymmetry of the electrostatic regulation. Because R764X and R766M were reported with CF patients (www.genet.sickkids.on.ca/cftr/app), we investigated if missense alanine mutation of R764 and R766 alters the channel processing and activity. Fig. 4D demonstrates that both R764A and R766A exhibited a normal channel density. Fig. 5 further shows that they were also normally processed. However, Fig. 6 demonstrates that the PKA-dependent activity of these two mutants was greatly enhanced. The K_{1/2} for PKA activation of R764A and R766A increased from 10 units/ml to 25 and 44 units/ml, respectively. Thus, a putative electrostatic attraction between R764 or R766 and D835, D836 or E838 may weaken the putative electrostatic attraction between K946 and D835, D836 or E838. To support this hypothesis, we determined if S832C is closed enough to S768C to form a detectable disulfide-bond crosslinking based on the Cys-free CFTR construct. Fig. 7 demonstrates that 10μM diamide greatly suppressed the channel activity of S768C/S832C/V510A mutant but failed to affect the channel activities of both S768C/V510A and S832C/V510A. Thus, the S768 phosphorylation site may be close to the C-terminal of NEG2. Taken together, these observations clearly suggest that a putative salt bridge between R766 or R764 and D836 or E838 may weaken the putative inhibitory electrostatic attraction between K946 and D835 or D836 or E838.

DISCUSSION

Since Ma’s group reported the regulation of PKA-dependent CFTR gating by NEG2 (19), it is obscure which part interacts with NEG2 mediating gating. Based on the findings that both S768 and D836 are close to CL3 (9-10), the current study further uncovered the regulation role of the complex electrostatic interaction between the R domain and CL3 in channel activation and processing. Our results strongly suggest that the putative electrostatic attractions of CL3 with the C-terminal of NEG2 in the whole CFTR prohibited the channel activation by PKA. More importantly, R764 and R766 of the S768 phosphorylation site may regulate the CL3-NEG2 interaction by a putative salt bridge with NEG2. Finally, the putative electrostatic attractions of CL3 with both NEG2 and the S768 phosphorylation site may lead to CFTR misprocessing.

Asymmetric electrostatic regulation of CFTR activation and processing - Unlike CFTR, other ABC transporters have not a regulatory domain but express and function well on the cell membrane. On the other hand, the deletion of the R domain does not affect the CFTR expression (10, 24-27). Moreover, the location of the R domain was found not to be essential for the function (28). Finally, the R domain is unstructured and disordered (29-30). Thus, the R domain seems unnecessary for the CFTR activation until Ma’s group found that a reduction in the negative charge of NEG2 of the R domain promotes the channel activation (19). Our study further suggests that the putative
electrostatic interactions between NEG2 and CL3 regulate the CFTR activation. First, the PKA sensitivity of channel activation was significantly enhanced for K946A, D835A, D836A and E838A mutants (Fig.2). Second, the curcumin sensitivity was also increased for K946A, K946D and D835R/D836R/E838R (Fig.3). Finally, it is very exciting that the putative electrostatic attraction between K946D/H950D and D835R/D836R/E838R dramatically suppressed the channel activity by stopping the channel processing or opening(10). However, the putative electrostatic attraction between K946/H950R and D835/D836/E838 failed to exert this inhibitory effect (Fig.4-5). Moreover, the putative Fe3+ bridge, H-bond, or S768 phosphorylation at the R-CL3 interface failed to affect this asymmetric electrostatic regulation. Therefore, the third charged motif may be involved.

The role of S768 phosphorylation site- Because both S768 and D836 are close to H950 (9, 10), and S768 may be closed to C832 (Fig.7), we examined if R764 or R766 interacts with NEG2 and thus destroys the symmetry of the putative electrostatic attraction between CL3 and NEG2. Our finding shows that R764A and R766A suppressed not the channel processing and the current density but the channel activation by PKA (Fig. 4-6). Thus, we propose two models of the electrostatic regulation. First, D835, D836 or E838 may interact electrostatically with both K946 and R764 or R766 (Fig. 8A). The former may prohibit the channel activation by PKA while the latter may promote the channel activation by PKA possibly by weakening the former interaction. On the other hand, the putative electrostatic attraction between CL3 and NEG2 may not be exchangeable. Otherwise, a putative electrostatic attraction of CL3 (K946D/H950D) with both NEG2 (D836R or E838R) and the S768 phosphorylation site (R764 or R766) may result in misprocessing and low channel activity (Fig.8B). Therefore, a complex electrostatic balance among CL3, NEG2 and the S768 phosphorylation site may ensure the normal channel processing and activity.

On the other hand, S945L and H949Y, found in patients with cystic fibrosis, are close to K946 and H950 of CL3 and stop maturation of the protein (31). What is more, CL3 also involves ubiquitination-dependent CFTR trafficking (32). Finally, D979A in CF patients causes misprocessing (33). Thus, the interaction of K946 with the COMMD1 protein (32) or D979 (33) may also result in the asymmetric electrostatic regulation by orchestrating the integral folding to enhance or stabilize CFTR expression on the membrane, allowing more proteins to exit from endoplasmic reticulum, or accelerating the fuse of stabilized CFTR-containing vesicles with the cell membrane.

Implication for CFTR activation by PKA phosphorylation- The important finding with NEG2 is that the deletion of NEG2 activates the CFTR channel in the presence of ATP but PKA does not continue to increase the channel activity (19). However, the current study indicates that mutation of D835, D836 and R838 to alaninne or arginine failed to promote the channel activation by ATP although the PKA-dependence was reduced. Thus, other parts of NEG2 may interact with other stimulatory RRxS motifs in the R domain. Phosphorylation of these sites or removal of those parts of NEG2 may weaken their electrostatic attractions and thus activate the channel.

REFERENCES


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The abbreviations used are: CF, cystic fibrosis, CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP-binding cassette; R, regulatory; TMD, transmembrane domain; NBD, nucleotide binding domain; PKA, protein kinase A; PKI, protein kinase inhibitory peptide; CL, cytoplasmic loop.

**FIGURE LEGENDS**

**Figure 1** CFTR schematic. A, The R domain is inserted between NBD1 and TMD2. Four cytoplasmic loops (CLs) are extended from TMD1 and TMD2. Residues at the interface of the R domain and CL3 are highlighted as red crosses. B, a putative model to define proximity relationships and the relative orientation of charged residues at the R-CL3 interface. Three helices are based on three reports (3, 19, 30). NEG2 is a small C-terminal segment (817-838) of the R domain and has a conserved helical region and a net charge of -9 (19).

**Figure 2** The PKA-dependent activity of CFTR mutants at the R-CL3 interface. A-C, Macroscopic currents across inside-out membrane patches excised from transfected HEK-293T cells expressing WT CFTR (A) and mutants K946A (B) and D836A (C) by using a ramp protocol (±80 mV). In the presence of 1.5mM ATP, PKA was added gradually until the current was no longer increased. The arrows indicate the final concentrations. 200µM glibenclamide or 10µM CFTRinh 172 were used to inhibit the channel activity. D, PKA titration curves for CFTR mutants at the R-CL3 interface (n=3-5). WT CFTR was a control.

**Figure 3** Activation of CFTR mutants at the R-CL3 interface by ATP and curcumin. Macroscopic currents across inside-out membrane patches excised from transfected HEK-293T cells expressing CFTR mutants K946A (A), K946D (B) and D835R/D836R/E838R (C) by using a ramp protocol (±80 mV). A-C, currents were activated with 1.5 mM ATP and 50µM curcumin followed by 6 units/ml PKA catalytic subunit but inhibited by 200µM glibenclamide or 10µM CFTRinh172. The arrows indicate the final concentrations. D, Relative current of CFTR mutants induced by ATP and curcumin at the R-CL3 interface. The relative activity of K946D was significantly smaller than that of D835R/D836R/E838R (n=3-4, *, P<0.05, unpaired t test).

**Figure 4** Effects of electrostatic interactions on current densities of CFTR mutants at the R-CL3 interface. Whole-cell currents from transfected HEK-293T cells expressing CFTR mutants D835R/D836R/E838R (A), H950R (B) and K946D/H950D/D835R/D836R/E838R (C) were recorded by using a ramp protocol (±40 mV). Cₘ=14.25pF, 16.84pF, and 24.64pF, respectively. The mutants were activated by 50µM forskolin and 50µM curcumin and inhibited by 200µM glibenclamide or 10µM CFTRinh172. (D) Mean whole-cell currents normalized to the cell capacitance (n=3-9). The current mediated by K946D/H950D/D835R/D836R/E838R was statistically smaller than the K946D/H950D and D835R/D836R/E838R and H950R currents (n=3-5, *, P < 0.05, unpaired t test). The currents mediated by S768D/K946D/H950D/D836R and S768D/K946D/H950D/E838R were statistically smaller than the S768D/K946D/H950D current (n=3, *, P < 0.05, unpaired t test).

**Figure 5** Effects of electrostatic interactions on the processing of CFTR mutants at the R-CL3 interface. Immunoblots indicate their relative intensities of immature Band B and mature Band C (n=4-7, *, P<0.05, unpaired t test).
Figure 6  The PKA-dependent activity of CFTR mutants R764A and R766A.  A, Unit channel currents across inside-out membrane patches excised from transfected HEK-293T cells expressing R766A by using a holding potential (+60 mV). In the presence of 1.5mM ATP, PKA was added gradually until the current was no longer increased. The arrows indicate the final concentrations. NEM was used to increase the channel activity (9). 200μM glibenclamide or 10μM CFTRinh172 were used to inhibit the channel activity.  B, PKA titration curves for CFTR mutants R764A and R766A (n=3-5). WT CFTR was a control.

Figure 7  Effects of diamide on CFTR mutants at the R-CL3 interface. Macroscopic currents across inside-out membrane patches excised from transfected HEK-293T cells expressing CFTR mutants S768C/V510A (A), S832C/V510A (B) and S768C/S832C/V510A (C) by using a ramp protocol (±80 mV). NPPB-AM is a CFTR-specific opener (25).  D, Fractional inhibition of the current by diamide for three CFTR mutants (n=3-5, *, p<0.05, unpaired t test).

Figure 8  Tentative electrostatic regulation mechanisms of CFTR activation and processing.  A, a putative electrostatic attraction between K946 and D835 or D836 or E838 may prevent the channel opening by PKA. On the other hand, a putative electrostatic attraction between R764 or R766 and D835 or D836 or E838 may promote the channel opening by weakening the putative NEG2-CL3 electrostatic attraction.  B, a putative electrostatic attraction of CL3 with both NEG2 and the S768 phosphorylation site may cause CFTR misprocessing.

Table 1  The whole-cell currents Im (pA) and capacitances Cm (pF) of HEK-293T cells expressing CFTR constructs in response to forskolin.

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<th>Control Cm</th>
<th>Stimulated Cm</th>
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Figure 3

A. Curcumin, ATP, PKA (U/ml), Glibenclamide, CFTRinh172

B. Curcumin, ATP, PKA (U/ml), CFTRinh172

C. Curcumin, ATP, PKA (U/ml), Glibenclamide, CFTRinh172

D. Bar graph showing the ratio of curcumin to PKA for K946A, K946D, K946D/H950D, D835R/D836R/E838R.
Figure 4

A) 

B) 

C) 

D) 

Expressed Current (pA/pF)
Figure 6

A

PKA (u/ml)

6 12 12 12 12 12

Glibenclamide

NEM

CFTRinh172

ATP

6

R766A

B

I/I\text{max} vs. PKA (Units/ml)

WT

R764A

R766A
Figure 7

A

PKI

Diamide (µM)

10 10 2 2 2

DTT (mM)

Glibenclamide

ATP/PKA

CFTRinh172

S768C/V510A

100s

100pA

S768C/V510A

S832C/V510A

100s

200pA

B

PKI

Diamide (10µM)

CFTRinh172

ATP/PKA

DTT (10mM)

Glibenclamide

S832C/V510A

200s

20pA

D

Fractional inhibition by diamide

S768C/V510A

S832C/S768C/V510A

S832C/V510A

0.6

0.4

0.2

0.0

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Regulation of Activation and Processing of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by a Complex Electrostatic Interaction between the Regulatory Domain and Cytoplasmic Loop 3
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