Functional Analysis of Mutations in the Putative Binding Site for Cystic Fibrosis Transmembrane Conductance Regulator Potentiators

INTERACTION BETWEEN ACTIVATION AND INHIBITION

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An increasing number of compounds able to potentiate the activity of mutants of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel have been identified by high throughput screening or by individual search of derivatives of known active compounds. Several lines of evidence suggest that most CFTR potentiators act through the same mechanism, probably by binding to the nucleotide binding domains to promote the activity of the protein and then, with lower affinity, to an inhibitory site. With the aim of identifying the activating binding site, we recently modeled the nucleotide binding domain dimer and predicted a common binding site for potentiators in its interface. To validate this model experimentally, we mutated some of the residues involved in the putative binding site, i.e. Arg553, Ala554, and Val1293. The activity of CFTR potentiators was measured as apical membrane currents on polarized cells stably expressing wild type or mutated proteins. CFTR activity was elicited by application of a membrane-permeable cAMP analogue following by increasing concentrations of potentiators. We found that all three mutants responded to cAMP, although the affinity of R553Q was higher than that of wild type CFTR. In R553Q and V1293G mutants, the dissociation constant of potentiators for the activating site was increased, whereas the dissociation constant for the inhibitory site was reduced. Our results show that the mutated residues are part of the activating binding site for potentiators, as suggested by the molecular model. In addition, these results suggest that the activating and inhibitory sites are not independent of each other.

Cystic fibrosis (CF), the most frequent lethal genetic disease in the Caucasian population, is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an anionic channel activated by PKA-dependent phosphorylation and gated by ATP. The life expectancy for people suffering from the disease has increased worldwide in the last decades because of earlier diagnosis, a more aggressive treatment of respiratory infections, and a better general care of patients. However, a specific treatment for CF has not been found thus far. The nearly 1000 mutations already found to cause CF may produce different functional defects, namely, (i) no protein synthesis, (ii) defective protein folding/trafficking, (iii) altered channel gating, (iv) reduced anion permeability, or (v) reduced protein amount. A single mutation, i.e. deletion of phenylalanine in position 508 (ΔPhe508), is present in at least one allele in 50–90% of CF patients. ΔPhe508 displays a severe trafficking defect with an almost absence of protein inserted in the plasma membrane. However, when the trafficking defect is corrected by incubation at low temperature, it also displays an altered gating (1, 2). Therefore, there is an enormous interest in finding chemical compounds able to correct class II and potentiate class III proteins. In addition to ΔPhe508, several other less frequent mutations cause class III defects. All of them produce an alteration of CFTR gating mechanisms that translates into a severely reduced open probability of the channel.

An increasing number of compounds able to activate class III CFTR mutants has been identified in the last 5 years by high throughput screening programs (1, 3–5) or by individual search of better derivatives/analogues of known active compounds (6–8). These compounds have been called potentiators for their ability to increase the response of the channel to cAMP-dependent phosphorylation. The potencies of the newly identified compounds are far better than those of classical potentiators such as genistein. In addition, analysis of the activity of similar structures to discriminate between active and inactive analogues, and pharmacokinetic analysis in animal models have already been done for some of them (3, 5, 6, 9). Nevertheless, the complete molecular structure of CFTR is still unknown, which precludes rational, mutation-specific drug design.

Several lines of evidence, based on protein-drug interactions, suggest that most CFTR potentiators act through the same mechanism. In fact, competition has been described between genistein and benzimidazolines (10), 7,8-benzoflavones and benzimidazolones (11), and genistein and capsaicin (12). Potentiators probably act by binding at the nucleotide binding domains (NBDs) to favor the chloride permeable state of the protein (13, 14). This hypothesis is supported by the observa-
tion that mutations in conserved residues of the NBDs such as G551D and G1349D exhibit a shift in the affinity for potentiators (5, 15–18). It is worth mentioning that several severe CF mutations occur within the NBDs. Binding of potentiators with lower affinity to a second site has been proposed as the cause of CFTR channel inhibition when these substances are applied at relatively high concentrations (13, 14, 19). With the aim of identifying the activating binding site of potentiators, we have recently modeled the NBD dimer (16) based on the crystal structure of human NBD1 (20, 21) using as template for the quaternary structure a homologous bacterial NBD dimer (22).

After in silico docking of several compounds, we compared the theoretical binding free energy measured on the model, with the experimental binding free energy obtained from dissociation constants from wild type, G551D, and G1349D proteins. We found a good correlation between these two parameters for the experimental binding free energy obtained from dissociation constants with wild type, G551D, and G1349D proteins. The relative position of mutated residues is shown in Fig. 1.

To gain further insight into potentiator binding, we investigated the effect of mutations of three residues predicted to form the binding site of CFTR potentiators, i.e. Arg553, Ala554, and Val1293 (see Fig. 1). We have measured the activity of three CFTR potentiators, namely genistein, Act-06 (23), and UCCF-029 (11), as apical membrane currents on cells stably expressing wild type or mutated proteins. CFTR activity was elicited by application of a submaximal concentration of the membrane-permeable cAMP analogue CPTcAMP followed by increasing concentrations of potentiators. We found that the stimulating effect of potentiators was reduced for R553Q and V1293G. Interestingly, we found a shift to the left of the inhibitory response to potentiators for the same mutants. Our results confirm that residues Arg553 and Val1293 may be involved in the activating binding site of potentiators, as suggested by our molecular model. In addition, these results suggest that activating and inhibitory actions are not independent of each other.

EXPERIMENTAL PROCEDURES

Mutants and Transfection—Mutations were introduced by recombinant PCR, and then the constructs were fully sequenced to verify the presence of the desired mutation and to ensure that other mutations had not been introduced. Mutant V1293G was introduced in a wild type CFTR construct contained in the expression vector pTracer-CMV (15) by a recombinant PCR method. For mutations A554E and R553Q, CFTR cDNA was subcloned on pcDNA3.1. Fisher rat thyroid (FRT) cells were stably transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. V1293G clones were selected and maintained in 800 μg/ml Zeocin, and A554E and R553Q constructs were selected and maintained in 1 mg/ml G418.

Cell Cultures—FRT cells expressing wild type (WT), V1293G, A554E, or R553Q CFTR were cultured on 60-mm Petri dishes with Con’s modified F12 containing 5% fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin and selection antibiotics, as described previously (15). For experiments, cells were seeded at high density (5 × 10⁶ cells/cm²) on Snapwell inserts (Costar, Corning) and maintained at 37 °C in a 5% CO₂, 95% air atmosphere. Apical and basolateral media were replaced every 48 h. Transepithelial resistance was daily measured with an epithelial voltmeter (Millipore-ERS, Millipore) using chopstick-like electrodes. After 6–7 days, FRT monolayers developed a transepithelial resistance in the range of 2–4 kilohms cm². Experiments were done at days 8–11 after seeding.

Electrophysiology—Permeable supports were mounted into an Ussing-like vertical diffusion chamber (Corning, Costar). The basolateral membrane of FRT epithelia was permeabilized with 250 μg/ml amphotericin B, and a transepithelial Cl⁻ gradient was applied as reported previously (15, 24). The apical chamber was bathed with a low Cl⁻-containing solution (in mM): 65 NaCl, 65 sodium gluconate, 1.5 KH₂PO₄, 2.7 KCl, 0.5 MgCl₂, 2 CaCl₂, 10 HEPES-Na, and 10 glucose (pH 7.4). The basolateral chamber was bathed instead with (in mM): 130 NaCl, 1.5 KH₂PO₄, 2.7 KCl, 0.5 MgCl₂, 1 CaCl₂, 10 HEPES-Na, and 10 glucose (pH 7.4). Membrane permeabilization was monitored by measuring the current response to a 10-mV stimulus. The resistance decreased progressively and reached a stable value in about 30 min; assumed that this was the condition for maximal permeabilization. Experiments were done at 37 °C, and solutions were bubbled with air. The transepithelial potential difference was short-circuited at 0 mV with a voltage clamp amplifier (DVC-1000, World Precision Instruments) connected to the chambers through Ag/AgCl electrodes and agar bridges. CFTR potentiators were added only to the apical side.

Analysis of Currents—We considered the effect of a potentiator as the current increase over the current elicited by 20 μM CPTcAMP. Because the apparent affinity for potentiators depends on CFTR phosphorylation by cAMP-dependent PKA (12, 16, 25), we also took this parameter into account in the
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fitting. Therefore, for each mutant we first studied the response to CPTcAMP. Dose-response relationships to the nucleotide were fitted with a rectangular hyperbolic function,

\[ \frac{I}{I_{\text{max}}} = \frac{[c]}{K_d + [c]} \]  

(Eq. 1)

The fraction of \( I_{\text{max}} \) obtained with 20 \( \mu M \) CPTcAMP was calculated and reported in Table 1 as \( I(20)/I(\text{max}) \). Next, to analyze the effect of potentiators, each dose-response relationship to the potentiators was first normalized to the expected response to 20 \( \mu M \) CPTcAMP. After normalization, potentiator dose-response curves were fitted to the following equation (25),

\[ I = \frac{([c][f_a(g) + K_a])}{K_a K_c + [c][f_c(g)] + [g] K_c + K_c K_a} \]  

(Eq. 2)

where \( c \) and \( g \) are the concentrations of CPTcAMP (20 \( \mu M \)) and potentiator, respectively, \( f_a \) is the fraction of current activated by the potentiator over the maximum asymptotic current obtained with CPTcAMP, \( K_d \) is the apparent dissociation constant for CPTcAMP, and \( K_a \) and \( K_c \) are the apparent dissociation constants for potentiator activation and inhibition sites, respectively. Data were fitted using the Levenberg-Marquardt algorithm, a form of nonlinear least squares fitting, as implemented in IgorPro (Wavemetrics).

Materials—CPTcAMP, amphotericin, and genistein were from Sigma. The potentiator 2-(4-pyridinium)benzo(h)AHC-9-chromen-4-one bisulfate, UCCF-029, and the inhibitor Inh172 (26) were purchased from Asinex. Potentiator Act-06 ((2Z,5E)5-(3,4-dimethoxybenzylidene)-2-[(4-hydroxyphenyl]imino]-1,3-thiazolidin-4-one) was purchased from ChemBridge.³

RESULTS

Response of CFTR mutants to phosphorylation by CPTcAMP—Potentiators are effective only on phosphorylated CFTR channels, and their effect is tightly correlated with the magnitude of CFTR activation by cAMP-dependent phosphorylation (11, 12, 25). Thus, before analyzing the affinity of potentiators for mutants, we first evaluated their response to phosphorylation by CPTcAMP, a membrane-permeable cAMP analogue. We measured CFTR activity as apical membrane currents on FRT cells stably expressing either wild type or mutant CFTRs. CPTcAMP at concentrations between 1 and 500 \( \mu M \), caused an increase in apical membrane currents (Fig. 2). Dose-response curves were fitted to Equation 1; the values obtained for the maximum current, \( I_{\text{max}} \), and affinity parameter, \( K_d \), are shown in Table 1. The apparent dissociation constant of CPTcAMP for mutants V1293G and A554E was not statistically different from that of the wild type protein (Fig. 2). Conversely, the dissociation constant for R553Q was significantly smaller. In fact, in this mutant \( K_d \) was 21.5 \( \mu M \), less than half the value of the wild type protein (54.2 \( \mu M \); see Table 1).

Response of CFTR Mutants to Genistein—To study the affinity of potentiators for different mutants, CFTR was first phosphorylated by using a CPTcAMP concentration that only partially activates the channel. In fact, on wild type cells a concentration of 20 \( \mu M \) produces a current increase corresponding to about one-third of the maximum current reachable

³ Potentiator names were kept as in the original references for clarity.

**FIGURE 2. Dose responses to CPTcAMP.** A, representative traces showing that increasing CPTcAMP concentrations cause a dose-dependent increase in apical currents on wild type and mutant CFTRs. In some experiments, the specific CFTR inhibitor, Inh172, was added at the end to confirm that all of the current elicited by the agonist was because of CFTR. CPTcAMP was added to the apical and basolateral chambers. B, normalized data are plotted against CPTcAMP concentration. Each symbol is the mean of 4–6 experiments, and vertical bars show S.E. In some cases, the error bars are within the symbol size. Continuous lines (WT, A554E, and V1293G) and broken lines (R553Q) indicate fitting of the data to Equation 1.

**TABLE 1** Parameters obtained from the fit to Equation 1 of CPTcAMP dose-response relationship on wild type and mutant CFTRs

For comparison, the parameters of G551D, a severe CF-causing mutation, are included.

|                      | Wild type | A554E | V1293G | R553Q | G551D |
|----------------------|-----------|-------|--------|-------|-------|
| \( I_{\text{max}} \) (\( \mu A/cm^2 \)) | 265.2 ± 13.5 | 66.9 ± 16.4* | 70.2 ± 14* | 93.8 ± 19* | 101.3 ± 2.8* |
| \( K_d \) (\( \mu M \))      | 54.2 ± 11.5 | 40.5 ± 5.9 | 48.4 ± 13.5 | 21.5 ± 4.5* | 74.2 ± 12.1 |
| \( I(20)/I(\text{max}) \)  | 0.3 ± 0.04 | 0.34 ± 0.03 | 0.32 ± 0.05 | 0.51 ± 0.05* | 0.23 ± 0.03 |

* \( p < 0.05. \)
with very high nucleotide concentrations (see \( I_{\text{max}} \) in Table 1). It is interesting to note that 20 \( \mu M \) CPTcAMP produced a similar current fraction (≈0.3) on mutants V1293G and A554E but half of the maximum current (≈0.5) on R553Q (see \( I/(20)/I(\text{max}) \) in Table 1), in agreement with the lower \( K_d \) of CPTcAMP found on this mutant.

Under this condition, increasing concentrations of genistein produced a dose-dependent current increase followed by inhibition at higher concentrations (Fig. 3A). Fig. 3, B–E, illustrates the response of the wild type and three mutants to different concentrations of genistein, as well as the data fitting to Equation 2, which describes the effects of a potentiator on cAMP-dependent chloride transport by CFTR. This equation is based on a four-state model for CFTR in the presence of saturating concentrations of ATP: one nonactive (nonphosphorylated) state; two conductive (phosphorylated via CPTcAMP) states, where the channel is either free or with a bound potentiator molecule; and one inhibited state with two bound potentiator molecules (25). Fitting genistein dose-response curves to Equation 2 yielded three parameters (see Table 2), the dissociation constants \( K_a \) and \( K_f \) for activation and inhibition, respectively, and \( f_{\text{max}} \), which describes the amount of extra current that the potentiator could activate over the maximum achievable by CPTcAMP alone (\( I_{\text{max}} \) Table 1). The response of mutant A554E to genistein, with almost no change on \( K_a \) or \( K_f \), was similar to the response of wild type CFTR. In contrast, the protein with the adjacent residue mutated, R553Q, behaved in a completely different way. Higher genistein concentrations were necessary to activate it, and conversely, lower concentrations were necessary to inhibit it, which resulted in a narrower range of action for the potentiator (Fig. 3D). Mutant V1293G behavior was between A554E and R553Q. In fact, the two equilibrium constants were modified but less than in mutant R553Q (Table 2).

**Response of CFTR Mutants to Other Potentiators**—To extend the previous observation to other potentiators, we repeated the stimulation of mutants using UCCF-029 (27) and Act-06 (23) instead of genistein. In general, for these compounds \( K_a \) changed on mutants in the same direction as \( K_a \) for genistein; however, the effect was less marked, except for mutant V1293G, which tended to be more sensitive to Act-06 (see Table 2). The dissociation constant \( K_f \) for UCCF-029 and Act-06 also followed the same pattern as for genistein. Arg<sup>553</sup> was particularly sensitive to the effect of the three potentiators studied for both activation and inhibition of CFTR.

It is important to note that although \( K_f \) of UCCF-029 was very low (in the nanomolar range) for all proteins, the \( K_f \) was very high, in the millimolar range. In general, for most CFTR potentiators, which are highly hydrophobic compounds, this represented a problem because their solubility was low, precluding a precise measurement of \( K_f \) for some CFTRs (see the standard errors in Table 2).

**Correlation between \( K_a \) and \( K_f \)**—As mentioned previously, genistein and UCCF-029 dissociation constants changed on mutants as compared with wild type CFTR. Notably, the change in \( K_f \) in the mutants seems to go along with a change in \( K_f \) but in the opposite direction. In fact, the more the \( K_a \) value shifted toward higher concentrations, as in the case of genistein for R553Q, the more the \( K_f \) value shifted toward lower concentrations. Conversely, when \( K_a \) was in the low concentration.
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![Graph showing correlation between Ka and Ki](Image)

**Figure 4.** Correlation between $K_a$ and $K_i$. This figure illustrates the spreading of $K_a$ versus $K_i$ for genistein (circles), Act-06 (squares), and UCCF-029 (diamonds). The CFTR proteins are indicated in red, yellow, green, and blue for R553Q, V1293G, A554E and WT, respectively. Each symbol is the mean of 4–6 experiments, and vertical and horizontal bars show S.E. The continuous line is the best fit of the logarithm of data points to a linear function.

range, as in the presence of UCCF-029, $K_i$ was very high. This inverse relationship between the two dissociation constants was observed for all phenotypes and for the three substances, as shown in the plot of $K_a$ against $K_i$ (see Fig. 4).

**DISCUSSION**

We have recently modeled the CFTR NBD1–NBD2 dimer to find out the putative binding site for the CFTR potentiators (16). By comparing the theoretical binding free energy on the model with the experimental binding free energy obtained from experimental dissociation constants, we identified a site located in the interface of the NBD dimer, strongly interacting with NBD1 and, to a lesser extent, with NBD2. To validate this site experimentally, we produced single-point mutations on residues predicted to form part of the potentiator binding site. We have recently modeled the CFTR NBD1–NBD2 dimer to experimentally, we produced single-point mutations on residues predicted to form part of the potentiator binding site. We have recently modeled the CFTR NBD1–NBD2 dimer to

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level modifies the apparent affinity of potentiators for CFTR (11, 25). We analyzed the mutant data within the framework of a recently proposed kinetic model (see “Experimental Procedures” and Ref. 25) that makes protein phosphorylation and the potentiator effect independent parameters. Thus, we fitted the potentiator dose–response relationship with Equation 2, a function that takes into account the phosphorylation level in terms of CFTR response to CAMP (or an analogue), assuming a saturating intracellular concentration of ATP (25). For each mutant, we first constructed the isothermal process as a function of the concentration of CPTcAMP in the presence of saturating ATP concentrations and, subsequently, the isothermal process as a function of the concentration of potentiator at a fixed concentration of CPTcAMP (20 μM). We did not expect to find that the amino acid substitutions changed the sensitivity of CFTR to CPTcAMP, because most PKA phosphorylation sites are located in the R domain and not in the NBDs (33). Surprisingly, we found a lower equilibrium constant for CPT-cAMP on mutant R553Q (see Table 2). Mutations of NBDs, like the CF mutation G551D (see Table 1 and Ref. 15) or the other mutations studied here, A554E and V1293G, do not seem to change significantly the sensitivity of the protein to CPTcAMP. One might explain the observed outcome by hypothesizing that by chance we have mutated a residue, Arg$^{553}$, that may be involved in the regulation of CFTR by phosphorylation. It is interesting to note that mutation R553Q is one of the CF mutations identified as ΔPhe$^{508}$ revertants (34). That means that introduction of R553Q mutation into human CFTR partially corrects the processing and gating defect caused by the ΔPhe$^{508}$ mutation, which has been suggested to modify the NBD1 surface that interacts with other CFTR domains (20).

The maximum current activated by CPT-cAMP was significantly lower in the three mutants with respect to the wild type protein. An even more marked reduction had been found previously for G551D (see Table 1 and Refs. 15 and 18), but in that case we measured the expression of both G551D and wild type proteins. We concluded that the small amount of G551D current was due to a severe gating defect of the mutant and not to reduced expression levels, as confirmed by the reduced open channel probability estimated from single channel recording. We have not examined the expression levels of the mutants studied here or their open probability; hence, a comparison with wild type expression is precluded. It is possible that the reduced current is in part due to lower levels of expression of the mutants.

Conversion of the Ala$^{554}$ residue to glutamic acid had little effect on the capability of potentiators to favor the conductive state, whereas conversion of Val$^{1293}$ to glycine increased the $K_a$ for genistein by 4-fold. Even more pronounced was the effect of the basic amino acid substitution on Arg$^{553}$ position. Its conversion to glutamine drastically increased genistein and UCCF-029 dissociation constants by 7-fold (Table 2). These results support the proposed molecular model of potentiator binding site and imply that the character of these amino acids is important for the effect of potentiators. In fact, the mutation that produced a stronger shift of the affinity for these compounds was R553Q, where the charge was eliminated. Actually, in the model, residues Arg$^{553}$ and Val$^{1293}$ have close interactions with
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potentiators (see Fig. 1; the distance between these amino acids and potentiators is less than 3.6 Å, whereas in A554E this distance is larger), and therefore a modification of any of them is predicted to produce a strong effect on the activation dissociation constant. The A554E mutant is different. Even if in the model residue Ala554 is positioned very near to the bound potentiator, it does not form a close interaction with the compound. Hence, the modification of this residue might not be relevant for potentiator binding. Similarly, the effect of these mutations does not depend on the fact of being localized on the NBDs, because ΔPhe508, which is on NBD1 but far away from the putative binding site for potentiators, displays an affinity for potentiators that is similar to that of the wild type protein (18).

The $K_a$ change on the mutants supports the idea that the binding site for potentiators is located in the interface between the two NBDs. In addition, our results reveal a previously unidentified characteristic of potentiators, an inverse relationship between $K_a$ and $K_i$. Actually, we found that an increased $K_a$ in R553Q and V1293G with respect to wild type CFTR is accompanied by a reduced $K_i$ (Figs. 3E and 4). In general, the inverse relationship between $K_a$ and $K_i$ on CFTR proteins suggests a close interaction between activation and inhibition of CFTR by potentiators, revealing that the binding sites for these two actions may not be independent, but most probably, they are correlated or even physically contiguous. In fact, our results indicate that when a compound is tightly bound to the activation site, it “disturbs” the inhibitory action. In contrast, a loosely bound potentiator probably does not stabilize the dimer and allows binding of another potentiator molecule that produces inhibition. As mentioned above, we found in the proposed NBD dimer model that the potentiator is placed in a cavity on NBD1 and interacts also with NBD2. It is possible that the potentiator interacts with each of the NBDs (before dimer formation) with different affinity. At lower concentrations, the compound possibly binds to one NDB favoring the dimeric conformation and consequently the conductive state. At higher concentrations, the potentiator might bind also to the other NBD. The presence of two bound potentiator molecules may preclude formation of the dimer. Probably, the NBD with higher affinity for potentiators is NBD1, because CF mutation G551D, situated on NBD1 near the potentiator binding site, causes a more pronounced effect on the equilibrium constant for the activation site than the symmetrical CF mutation on NBD2, G1349D (16). Although an unequivocal quantitative evaluation cannot be made on the molecular model, we could hypothesize that the potentiator creates more contacts with NBD1 than with NBD2 (see Fig. 1). An alternative explanation is that the binding site might be unique and that the activating and inhibitory effects depend on how the potentiator molecule is accommodated in the cavity between the NBDs. Undoubtedly, more studies will be required to better understand the relationship between potentiator dissociation constants and to identify the inhibitory site.

In conclusion, we believe that both the molecular model of the potentiator binding site (16) and the results presented here and obtained on mutants of this putative site strongly support the idea that CFTR potentiators bind in the NBD interface in close contact with the mutated amino acids, in particular with residues Arg553 and Val1293. Besides the impact of these results on the understanding of how potentiators bind to CFTR, our data have an interesting implication that must be further investigated. If an inverse correlation between $K_a$ and $K_i$ is demonstrated to be a general feature of all potentiators, then a high affinity compound used to treat CF patients virtually would not cause inhibition at high doses.

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