THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 12, pp. 9098–9104, March 23, 2007

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

*INTERACTION BETWEEN ACTIVATION AND INHIBITION**

Received for publication, December 13, 2006, and in revised form, January 22, 2007 Published, JBC Papers in Press, January 23, 2007, DOI 10.1074/jbc.M611411200

Olga Zegarra-Moran[‡1], **Martino Monteverde**[‡], **Luis J. V. Galietta**[‡], **and Oscar Moran**[§]

From the [‡]*Laboratorio di Genetica Molecolare, Istituto G. Gaslini, Largo Gerolamo Gaslini 5, I-16148 Genova, Italy and the* [§]*Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Via De Marini 6, I-16149 Genova, Italy*

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.* Arg^{553}, Ala^{554}, and Val^{1293}. 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 followed 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),[2] 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 (ΔPhe^{508}), is present in at least one allele in 50–90% of CF patients. ΔPhe^{508} 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 ΔPhe^{508}, 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 benzimidazolones (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-

* This work was supported by grants from the Italian Cystic Fibrosis Foundation (FFC 6/2003 and FFC 4/2006). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 390105636403; Fax: 390103779797; E-mail: ozegarra@unige.it.

[2] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PKA, cAMP-dependent protein kinase; CPTcAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; NBD, nucleotide binding domain; FRT, Fisher rat thyroid; WT, wild type.

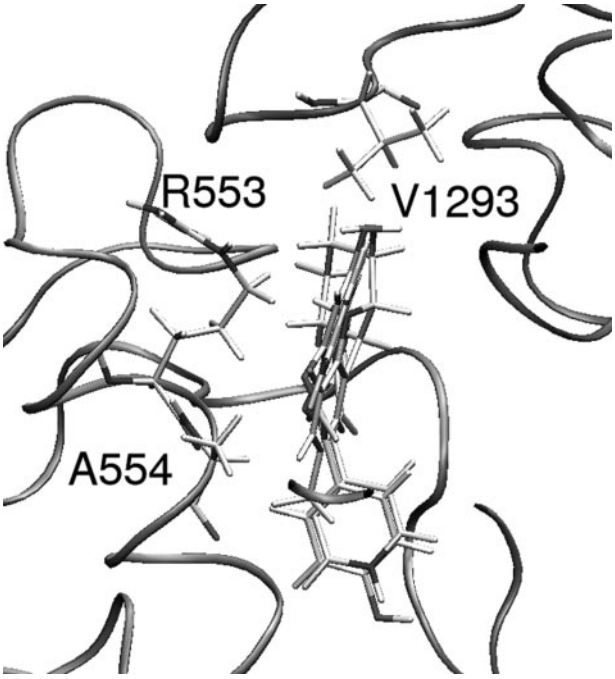

FIGURE 1. **Relative position of mutated residues.** The relative position of the CFTR potentiators and amino acids mutated on the putative binding site for potentiators was obtained from the model described in Moran *et al.* (16). Genistein, UCCF-029, and Act-06 are superposed.

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 a putative binding site located in the interface of the NBD1–NBD2 dimer, embedded in a cavity on NBD1, and interacting also with the NBD2 surface.

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.* Arg^{553}, Ala^{554}, and Val^{1293} (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 the 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 Arg^{553} and Val^{1293} 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 Coon'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 ($5{\cdot}10^5$cells/cm^2) on Snapwell inserts (Costar, Corning) and maintained at 37 °C in a 5% CO_2, 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 cm^2. 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 KH_2PO_4, 2.7 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES-Na, and 10 glucose (pH 7.4). The basolateral chamber was bathed instead with (in mM): 130 NaCl, 1.5 KH_2PO_4, 2.7 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 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

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,

$$\frac{I}{I_{max}} = \frac{[c]}{K_d + [c]} \quad \text{(Eq. 1)}$$

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

$$I = \frac{([c](f_A[g] + K_a)K_i}{K_dK_aK_i + [c]([g]^2 + [g]K_i + K_aK_i)} \quad \text{(Eq. 2)}$$

where c and g are the concentrations of CPTcAMP (20 μM) 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_i 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*)4*H*-chromen-4-one bisulfate, UCCF-029, and the inhibitor Inh172 (26) were purchased from Asinex. Potentiator Act-06 ((2*Z*,5*E*)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 μM, caused an increase in apical membrane currents (Fig. 2*A*). Dose-response curves were fitted to Equation 1; the values obtained for the maximum current, I_{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 μM, less than half the value of the wild type protein (54.2 μM; 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 μM produces a current increase corresponding to about one-third of the maximum current reachable

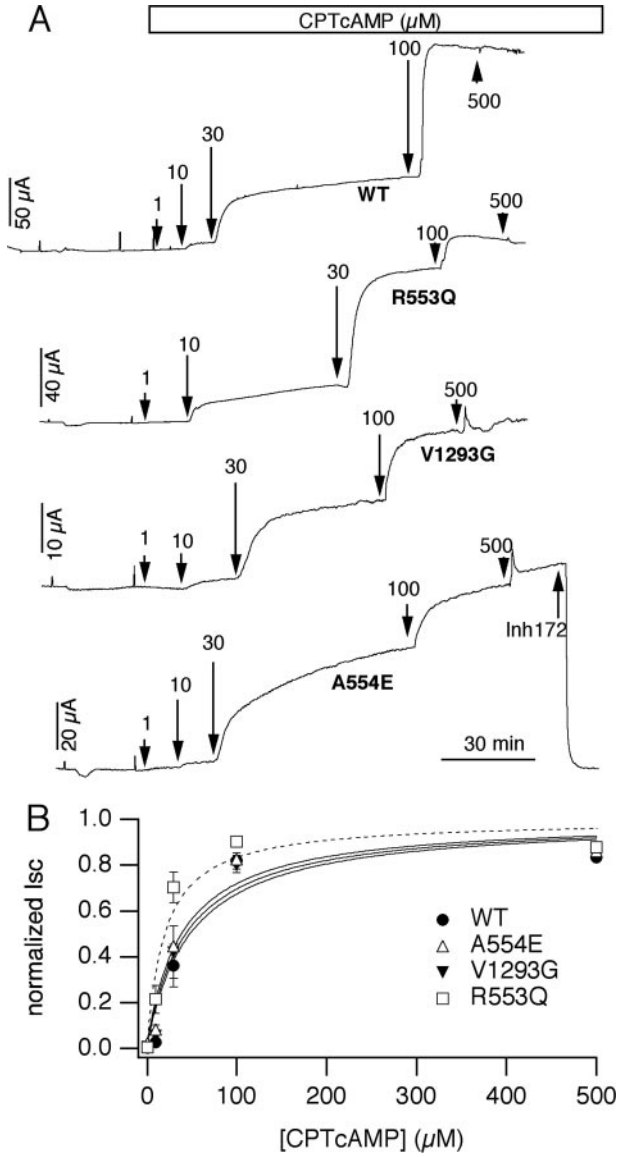

FIGURE 2. **Dose responses to CPTcAMP.** *A*, representative traces showing that increasing CPTcAMP concentrations cause a dose-dependent increase in apical currents on wild type and mutant CFTRs. In some experiments, the specific CFTR inhibitor, Inh172, was added at the end to confirm that all of the 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
	$n = 5$	$n = 4$	$n = 4$	$n = 6$	$n = 7$
I_{max} (μA/cm^2)	282.2 ± 13.5	66.9 ± 16.4[a]	70.2 ± 14[a]	93.8 ± 19[a]	10.1 ± 2.8[a]
K_d (μM)	54.2 ± 11.5	40.5 ± 5.9	48.4 ± 13.5	21.5 ± 4.5[a]	74.2 ± 12.1
$I(20)/I(max)$	0.3 ± 0.04	0.34 ± 0.03	0.32 ± 0.05	0.51 ± 0.05[a]	0.23 ± 0.03

[a] $p < 0.05$.

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

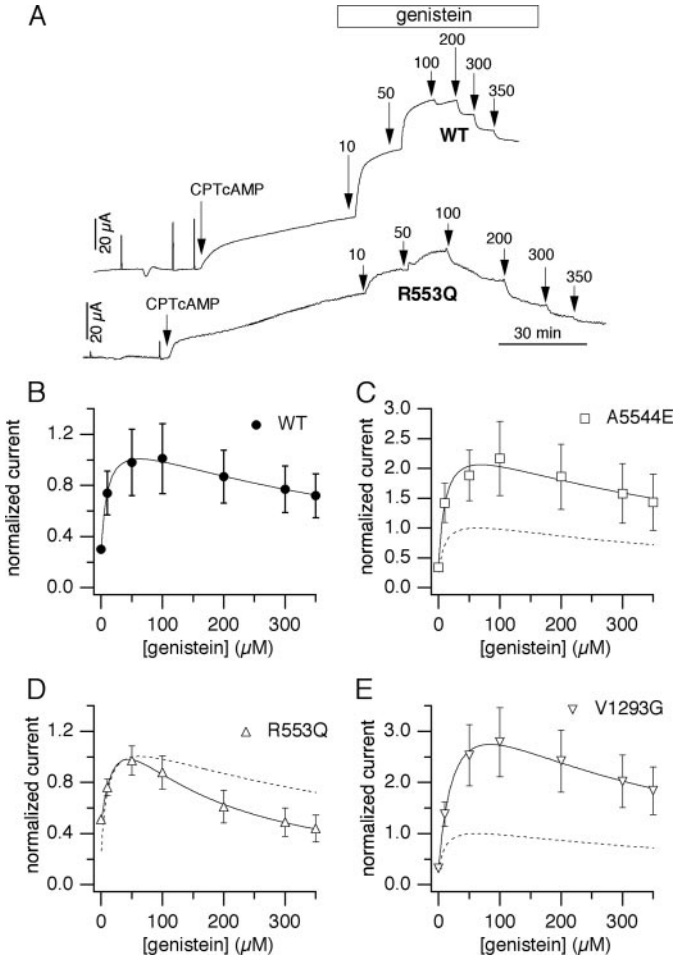

FIGURE 3. **Genistein dose-response relationships.** *A*, representative traces showing the response of wild type CFTR and mutant R553Q to application of genistein. CPTcAMP and genistein were added where indicated by *arrows*. *B*, dose-response relationship to genistein of wild type CFTR. Data were first normalized to the maximum effect and then averaged. *C–E*, comparison of normalized and averaged genistein dose-response relationships of mutants V1293G, A554E, and R553Q (see *symbol keys*) to WT (*dashed lines*). Each *symbol* is the mean of 4–6 experiments, and *vertical bars* show S.E. *Continuous* and *dashed lines* indicate fitting of the data to Equation 2.

with very high nucleotide concentrations (see I_{max} in Table 1). It is interesting to note that 20 μM CPTcAMP produced a similar current fraction (~0.3) on mutants V1293G and A554E but half of the maximum current (~0.5) on R553Q (see $I(20)/I(max)$ 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. 3*A*). 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_i for activation and inhibition, respectively, and f_A, which describes the amount of extra current that the potentiator could activate over the maximum achievable by CPTcAMP alone (I_{max}, Table 1). The response of mutant A554E to genistein, with almost no change on K_a or K_i, 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. 3*D*). Mutant V1293G behavior was between A554E and R553Q. In fact, the two equilibrium constants were modified but less than in mutant R553Q (Table 2).

TABLE 2

Parameters obtained from potentiator dose-response relationships

The curves were first normalized to the response to 20 μM CPTcAMP and then fitted to Equation 2.

Compounds	Protein	*n*	f_A	K_a	K_i
				μM	μM
Genistein	Wild type	5	1.33 ± 0.41	3.08 ± 0.74	562.4 ± 111.1
	A554E	6	2.75 ± 0.62	4.58 ± 0.51	408.6 ± 84.8
	V1293G	6	4.87 ± 1.91	12 ± 3.9[a]	270.5 ± 36.4[a]
	R553Q	6	1.87 ± 0.12	22.28 ± 5.2[a]	119.9 ± 34.9[a]
UCCF029	Wild type	4	0.41 ± 0.04	0.021 ± 0.002	1036 ± 523
	A554E	4	1.56 ± 0.45[a]	0.036 ± 0.009	1519 ± 651
	V1293G	5	3.19 ± 1.09	0.042 ± 0.008	843 ± 126
	R553Q	5	1.08 ± 0.17[a]	0.154 ± 0.029[a]	547 ± 99
Act-06	Wild type	5	0.8 ± 0.17	0.69 ± 0.33	439.6 ± 108
	A554E	4	1.67 ± 0.42	0.74 ± 0.25	319.1 ± 42.4
	V1293G	4	1.25 ± 0.16	0.35 ± 0.09	383.8 ± 28.9
	R553Q	6	1.77 ± 0.47[a]	2.11 ± 0.73	179.1 ± 73.2[a]

[a] Student's *t* test indicated that these values were statistically different from those on WT CFTR with $p < 0.05$.

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_i for UCCF-029 and Act-06 also followed the same pattern as for genistein. Arg^{553} 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_a of UCCF-029 was very low (in the nanomolar range) for all proteins, the K_i 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_i for some CFTRs (see the standard errors in Table 2).

Correlation between K_a and K_i—As mentioned previously, genistein and UCCF-029 dissociation constants changed on mutants as compared with wild type CFTR. Notably, the change in K_a in the mutants seems to go along with a change in K_i 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_i value shifted toward lower concentrations. Conversely, when K_a was in the low concentration

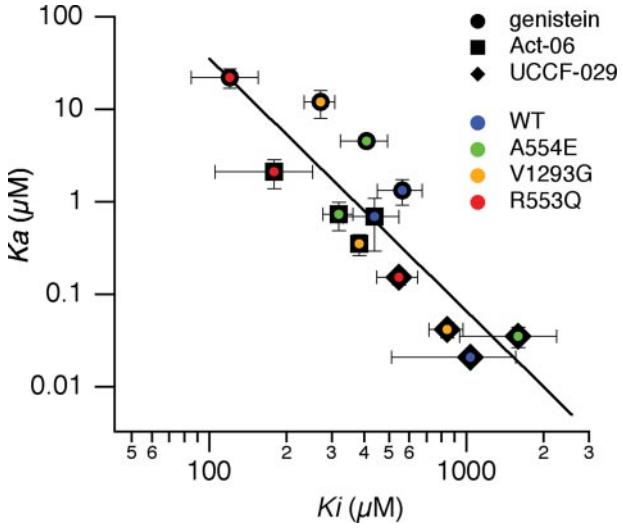

FIGURE 4. **Correlation between K_a and K_i.** This figure illustrates the spreading of K_a *versus* K_i for genistein (*circles*), Act-06 (*squares*), and UCCF-029 (*diamonds*). The CFTR proteins are indicated in *red*, *yellow*, *green*, and *blue* for R553Q, V1293G, A554E and WT, respectively. Each *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 selected three positions, two near the LSGGQ signature in NBD1 (Ala^{554} and Arg^{553}) and one in the Q-loop of NBD2 (Val^{1293}). Residues 553 and 554 form part of the cavity on NBD1 that, in our model, is occupied by the potentiators, whereas residue 1293 is on the surface of NBD2 that is in contact with the potentiator. We completely changed the characteristic of the charged amino acid in position 553 by substituting the basic arginine with glutamine. In the same way, in position 554 we introduced an acidic amino acid by replacing the aliphatic alanine with glutamic acid. Finally, in position 1293 with did a more conservative substitution by changing glycine for valine, both being small aliphatic residues.

The CFTR Cl^- channel is gated by ATP binding and hydrolysis at the NBDs (28–31), but ATP is not sufficient to activate the channel. Activation is obtained after PKA-mediated phosphorylation of the R (regulatory) domain, probably followed by a structural change necessary to allow CFTR to start the gating cycle (29, 32, 33). This implies that an interaction between the R domain and the NBDs begins or ends to permit the channel to open. To make this picture more complex, the phosphorylation 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 CPTcAMP 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 CPTcAMP 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

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 Ala^{554} 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 ΔPhe^{508}, 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. 3*E* 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 Arg^{553} and Val^{1293}. 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.

Acknowledgments—We are grateful to Dr. Nicoletta Pedemonte for help in the screening of mutant clones and to Dr. Chiara Pincin, who produced the cDNA constructs with the three point mutations.

REFERENCES

1. Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J., and Verkman, A. S. (2005) *J. Clin. Investig.* **115,** 2564–2571
2. Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R. G., Pavirani, A., Lecocq, J. P., and Lazdunski, M. (1991) *Nature* **354,** 526–528
3. Yang, H., Shelat, A. A., Guy, R. K., Gopinath, V. S., Ma, T., Du, K., Lukacs, G. L., Taddei, A., Folli, C., Pedemonte, N., Galietta, L. J., and Verkman, A. S. (2003) *J. Biol. Chem.* **278,** 35079–35085
4. Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L. R., Miller, M., Neuberger, T., Olson, E., Panchenko, V., Rader, J., Singh, A., Stack, J. H., Tung, R., Grootenhuis, P. D., and Negulescu, P. (2006) *Am. J. Physiol.* **290,** L1117–L1130
5. Pedemonte, N., Sonawane, N. D., Taddei, A., Hu, J., Zegarra-Moran, O., Suen, Y. F., Robins, L. I., Dicus, C. W., Willenbring, D., Nantz, M. H., Kurth, M. J., Galietta, L. J., and Verkman, A. S. (2005) *Mol. Pharmacol.* **67,** 1797–1807
6. Marivingt-Mounir, C., Norez, C., Derand, R., Bulteau-Pignoux, L., Nguyen-Huy, D., Viossat, B., Morgant, G., Becq, F., Vierfond, J. M., and Mettey, Y. (2004) *J. Med. Chem.* **47,** 962–972
7. Cai, Z., and Sheppard, D. N. (2002) *J. Biol. Chem.* **277,** 19546–19553
8. Cai, Z., Taddei, A., and Sheppard, D. N. (2006) *J. Biol. Chem.* **281,** 1970–1977
9. Springsteel, M. F., Galietta, L. J., Ma, T., By, K., Berger, G. O., Yang, H., Dicus, C. W., Choung, W., Quan, C., Shelat, A. A., Guy, R. K., Verkman, A. S., Kurth, M. J., and Nantz, M. H. (2003) *Bioorg. Med. Chem.* **11,** 4113–4120
10. Al-Nakkash, L., Hu, S., Li, M., and Hwang, T. C. (2001) *J. Pharmacol. Exp. Ther.* **296,** 464–472
11. Caci, E., Folli, C., Zegarra-Moran, O., Ma, T., Springsteel, M. F., Sammelson, R. E., Nantz, M. H., Kurth, M. J., Verkman, A. S., and Galietta, L. J. (2003) *Am. J. Physiol.* **285,** L180–L188
12. Ai, T., Bompadre, S. G., Wang, X., Hu, S., Li, M., and Hwang, T. C. (2004) *Mol. Pharmacol* **65,** 1415–1426
13. Weinreich, F., Wood, P. G., Riordan, J. R., and Nagel, G. (1997) *Pfluegers Arch. Eur. J. Physiol.* **434,** 484–491
14. Wang, F., Zeltwanger, S., Yang, I. C.-H., Nairn, A. C., and Hwang, T.-C. (1998) *J. Gen. Physiol.* **111,** 477–490
15. Zegarra-Moran, O., Romio, L., Folli, C., Caci, E., Becq, F., Vierfond, J. M., Mettey, Y., Cabrini, G., Fanen, P., and Galietta, L. J. (2002) *Br. J. Pharmacol.* **137,** 504–512
16. Moran, O., Galietta, L. J. V., and Zegarra-Moran, O. (2005) *Cell. Mol. Life Sci.*, **62,** 446–460
17. Derand, R., Bulteau, L., Mettey, Y., Zegarra-Moran, O., Howell, D., Randak, C., Galietta, L. J. V., Cohn, J. A., Norez, C., Romio, L., Vierfond, J.-M., Joffre, M., and Becq, F. (2001) *Am. J. Physiol.* **281,** C1657–C1666
18. Pedemonte, N., Diena, T., Caci, E., Nieddu, E., Mazzei, M., Ravazzolo, R., Zegarra-Moran, O., and Galietta, L. J. (2005) *Mol. Pharmacol.* **68,** 1736–1746
19. Lansdell, K. A., Cai, Z., Kidd, J. F., and Sheppard, D. N. (2000) *J. Physiol.* (*Lond.*) **524,** 317–330
20. Lewis, H. A., Zhao, X., Wang, C., Sauder, J. M., Rooney, I., Noland, B. W., Lorimer, D., Kearins, M. C., Conners, K., Condon, B., Maloney, P. C.,

Guggino, W. B., Hunt, J. F., and Emtage, S. (2005) *J. Biol. Chem.* **280,** 1346–1353
21. Thibodeau, P. H., Brautigam, C. A., Machius, M., and Thomas, P. J. (2005) *Nat. Struct. Mol. Biol.* **12,** 10–16
22. Yuan, Y. R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P. J., and Hunt, J. F. (2001) *J. Biol. Chem.* **276,** 32313–32321
23. Ma, T., Vetrivel, L., Yang, H., Pedemonte, N., Zegarra-Moran, O., Galietta, L. J., and Verkman, A. S. (2002) *J. Biol. Chem.* **277,** 37235–37241
24. Sheppard, D. N., Rich, D. P., Ostedgaard, L. S., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1993) *Nature* **362,** 160–164
25. Moran, O., and Zegarra-Moran, O. (2005) *FEBS Lett.* **579,** 3979–3983
26. Taddei, A., Folli, C., Zegarra-Moran, O., Fanen, P., Verkman, A. S., and Galietta, L. J. V. (2004) *FEBS Lett.* **558,** 52–56
27. Galietta, L. J., Springsteel, M. F., Eda, M., Niedzinski, E. J., By, K., Haddadin, M. J., Kurth, M. J., Nantz, M. H., and Verkman, A. S. (2001) *J. Biol. Chem.* **276,** 19723–19728
28. Vergani, P., Nairn, A. C., and Gadsby, D. C. (2003) *J. Gen. Physiol.* **121,** 17–36
29. Mense, M., Vergani, P., White, D. M., Altberg, G., Nairn, A. C., and Gadsby, D. C. (2006) *EMBO J.* **25,** 4728–4739
30. Aleksandrov, L., Aleksandrov, A. A., Chang, X. B., and Riordan, J. R. (2002) *J. Biol. Chem.* **277,** 15419–15425
31. Vergani, P., Lockless, S. W., Nairn, A. C., and Gadsby, D. C. (2005) *Nature* **433,** 876–880
32. Tabcharani, J. A., Chang, X. B., Riordan, J. R., and Hanrahan, J. W. (1991) *Nature* **352,** 628–631
33. Gadsby, D. C., and Nairn, A. C. (1999) *Physiol. Rev.* **79,** Suppl. 1, S77–S107
34. Teem, J. L., Berger, H. A., Ostedgaard, L. S., Rich, D. P., Tsui, L. C., and Welsh, M. J. (1993) *Cell* **73,** 335–346

Functional Analysis of Mutations in the Putative Binding Site for Cystic Fibrosis Transmembrane Conductance Regulator Potentiators: INTERACTION BETWEEN ACTIVATION AND INHIBITION

Olga Zegarra-Moran, Martino Monteverde, Luis J. V. Galietta and Oscar Moran

J. Biol. Chem. 2007, 282:9098-9104.
doi: 10.1074/jbc.M611411200 originally published online January 23, 2007
