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ORIGINAL PAPER

A stable ATP binding to the nucleotide binding domain is important for reliable gating cycle in an ABC transporter CFTR

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Abstract Cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, a member of ABC transporter superfamily, gates following ATP-dependent conformational changes of the nucleotide binding domains (NBD). Reflecting the hundreds of milliseconds duration of the channel open state corresponding to the dimerization of two NBDs, macroscopic WT-CFTR currents usually showed a fast, single exponential relaxation upon removal of cytoplasmic ATP. Mutations of tyrosine1219, a residue critical for ATP binding in second NBD (NBD2), induced a significant slow phase in the current relaxation, suggesting that weakening ATP binding affinity at NBD2 increases the probability of the stable open state. The slow phase was effectively diminished by a higher affinity ATP analogue. These data suggest that a stable binding of ATP to NBD2 is required for normal CFTR gating cycle, andthat the instability of ATP binding frequently halts the gating cycle in the open state presumably through a failure of ATP hydrolysis at NBD2.

Keywords CFTR · ABC transporter · ATP hydrolysis · Gating · Anion channel

Introduction

Cystic fibrosis transmembrane conductance regulator CFTR (ABCC7), a member of the ABC transporter superfamily, functions as a chloride channel [1, 2]. In addition to the pore-forming membrane spanning domains, CFTR has two nucleotide-binding domains, NBD1 and NBD2, containing the prototypical Walker A and Walker B motifs that interact with ATP. Opening of the CFTR chloride channel is associated with dimerization of CFTR's two NBDs [3] in a head-to-tail configuration seen in the crystal structures of numerous ABC proteins [4–6].

Because ATP binding leads to CFTR channel opening, CFTR can be considered as a ligand-gated channel operationally [7]. For ligand-gated channels in general, ligand binding shifts the gating equilibrium in favor of the open state, whereas ligand dissociation promotes channel closing. We have previously shown that ATP binding to NBD2, but not NBD1, is critical for catalyzing channel opening ([8–11] (but cf. [12]). Unlike other classical ligand-gated channels, however, the bound ligand for CFTR is consumed. It is believed that ATP hydrolysis initiates the separation of the NBD dimer and thereby closes the channel [7, 13]. Biochemical studies showed that NBD2,

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but not NBD1, hydrolyzes ATP [14–16]. Because of a fast rate of ATP hydrolysis in wild-type (WT)-CFTR, the single-channel mean open time and the time constant for macroscopic current relaxation upon washing out ATP are of the order of hundreds of milliseconds [12]. Contrary to WT-CFTR, CFTR mutants whose ATP hydrolysis at NBD2 is abolished (i.e., E1371S, K1250A), can remain open for minutes [9, 12, 17–20].

We have previously reported that mutations at tyrosine 1219, a conserved aromatic amino acid in NBD2, induce a drastic rightward shift of the ATP dose–response relationship [21]. Using single channel recording, the Y1219 mutation-induced change of the ATP binding affinity was characterized by an increase of closed time (decrease of opening rate) with little change of mean open time (closing rate) [21], which suggested that the Y1219 mutations mainly affected the ATP binding affinity to ABP2. The degree of dose–response shift, a descending order of Y1218G, Y1219I, Y1219F and Y1219W, seemed to depend on the similarity of chemical properties, e.g., hydrophobicity and π bond by aromatic ring, of the side chains introduced by the mutations to tyrosine. Thus, the more 'preserved' the mutation, the less the effect on the dose–response relationship, suggesting a graded change of the binding affinity for ATP by different mutations.

However, interestingly, in macroscopic currents obtained from inside-out patch expressing Y1219G-CFTR, we have observed a significant slow component in the current relaxation after ATP washout. This suggests that a fraction of Y1219G-CFTR channels in the patch exhibited long-lasting openings after ATP washout whereas the remaining majority of Y1219G-CFTR channels closed within hundreds of milliseconds as WT-CFTR.

In this study, to investigate the possible mechanism underlying this phenomena, we characterized the current relaxation after ATP washout of CFTR channels carrying mutations at the Y1219 residue and discussed the underlying mechanism by using a Monte Carlo simulation.

Materials and methods

Cell culture and transient expression of CFTR

WT- and mutant CFTR channels were expressed transiently in Chinese hamster ovary (CHO) cells grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. The cDNA constructs were cotransfected with pEGFP-C3 (Clontech, Palo Alto, CA, USA) with the SuperFect transfection reagent (Qiagen, Valencia, CA, USA). The transfected cells were split between two 35-mm tissue culture dishes containing sterilized glass chips for patch-clamp studies.

Electrophysiological recordings

Inside-out patch-clamp recordings were performed at room temperature. The pipette solution contained (in mM) 140 NMDG-Cl, 2 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES (pH 7.4 with NMDG). Initially cells were perfused with a bath solution containing 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 5 HEPES, and 20 sucrose (pH 7.4 with NaOH). After the establishment of an inside-out configuration, the patch was perfused with a standard perfusion solution (i.e., intracellular solution) containing 150 NMDG-Cl, 2 $MgCl_2$, 10 EGTA, and 8 Tris (pH 7.4 with NMDG). Patches were held at -60 mV in all experiments. At this membrane potential, downward deflections represent channel opening. However, we inverted the current direction for data presentation. CFTR channels were activated by a combined application of 25 U/ml cAMP-dependent protein kinase (PKA) catalytic subunits and 1 mM MgATP. Once activated, the CFTR channels were opened by an application of 5 mM MgATP. Single channel and macroscopic currents were measured with 50-fold amplification for single channel and 1- to 20-fold for macroscopic current recordings, then filtered at 100 Hz and digitized at 500 Hz using an EPC10 patch-clamp amplifier (HEKA-Electronics, Lambrecht/Pfalz, Germany).

A fast solution exchange system SF-77B (Perfusion Fast-Step; Warner Instrument, Hamden, CT, USA) that can switch the perfusion solutions completely within 20 ms was used to allow accurate estimation of the relaxation time constant [22].

MgATP was purchased from Sigma (St. Louis, MO, USA) and N^6-(2-phenyltehyl)-ATP (P-ATP) was purchased from Biolog Life Science Institute (Bremen, Germany). PKA was purchased from Promega (Madison, WI, USA). All nucleotides were dissolved in the perfusion solution for the experiments and pH was adjusted 7.4 with NMDG.

Data analysis

The macroscopic current relaxations were analyzed by using the Igor Pro Program (Wavemetrics, Portland, OR, USA). The relaxation time constant and the fraction of each relaxation component were determined by fitting the current decay with a single or double exponential function. Data were presented as mean ± SEM; n represents the number of experiments. Unpaired Student's t test was performed for comparing data obtained from relaxation analyses (Fig. 2) using SigmaPlot 8.0 (Systat Software, San Jose, CA, USA) and $P < 0.05$ was considered significant.

Monte Carlo gating simulation

To quantitatively evaluate the proposed gating model (Fig. 6), we performed a Monte Carlo simulation of CFTR

channel gating, in which open and closed intervals were generated from the kinetic models to reproduce the macroscopic current relaxation after ATP washout (Fig. 7). The Monte Carlo simulation model reproduced a current relaxation mimicking the Y1219 mutation-induced slow relaxation in the experiments. The time constant and the relative amplitude of the slow component were determined with a double exponential fit. The data were summarized into the average and SEM of 10 trials with different random seed numbers. Kinetic parameters (rate constants) used for simulations were extracted from previously published papers or derived from our present experimental results (see "Results" and "Discussion" for more detail).

All the computer programs were homemade and written in FORTRAN, compiled by a FORTRAN compiler (Microsoft, Redmond, WA, USA) and run under the WINDOWS XP operating system (Microsoft) in a personal computer equipped with an Intel Core 2 Duo processor (Dell, Round Rock, TX, USA).

Results

Figure 1a shows a representative WT-CFTR macroscopic current relaxation after a rapid removal of ATP. For WT-CFTR, removal of ATP in excised inside-out patches usually resulted in a fast current decay with a relaxation time constant of 645 ± 80 ms ($n = 9$) (Fig. 1a, gray dashed line in the enlarged inset) whereas it occasionally contained a very small and slow additional component possibly underlied by long-lasting opened WT-CFTR channels (see Fig. 5, below). At the single channel level, ATP removal usually induced an immediate closing on WT-CFTR channels (Fig. 1b). These macroscopic and microscopic kinetic data indicate that WT-CFTR basically has a one open state with a time constant around hundreds of milliseconds as reported previously [e.g., 12, 20].

Effects of Y1219 mutations on open state of CFTR channels

Our previous report [21] identified two aromatic amino acids (W401 in NBD1 and Y1219 in NBD2) that play a critical role in stabilizing ATP binding in its respective binding site. We have also characterized a series of Y1219 mutations that cause different degree of alterations in ATP binding affinity. We next examined the macroscopic current relaxation in inside-out patches containing those mutants.

Indeed, the current decay upon washout of ATP for the Y1219G mutants consistently followed a biphasic time course with a visible slow component (Fig. 2a). In contrast, the current relaxation for the W401G mutants showed a major monotonic fast decay which was followed by some minimal residual channel activity (Fig. 2a). Figure 2b compares macroscopic current relaxations for Y1219G, Y1219I, Y1219F and Y1219W mutants. Although a slow phase of current decay can be seen with Y1219G and Y1219I, this second phase is hardly discernable for the Y1219W mutant. The result with the Y1219F mutant lies

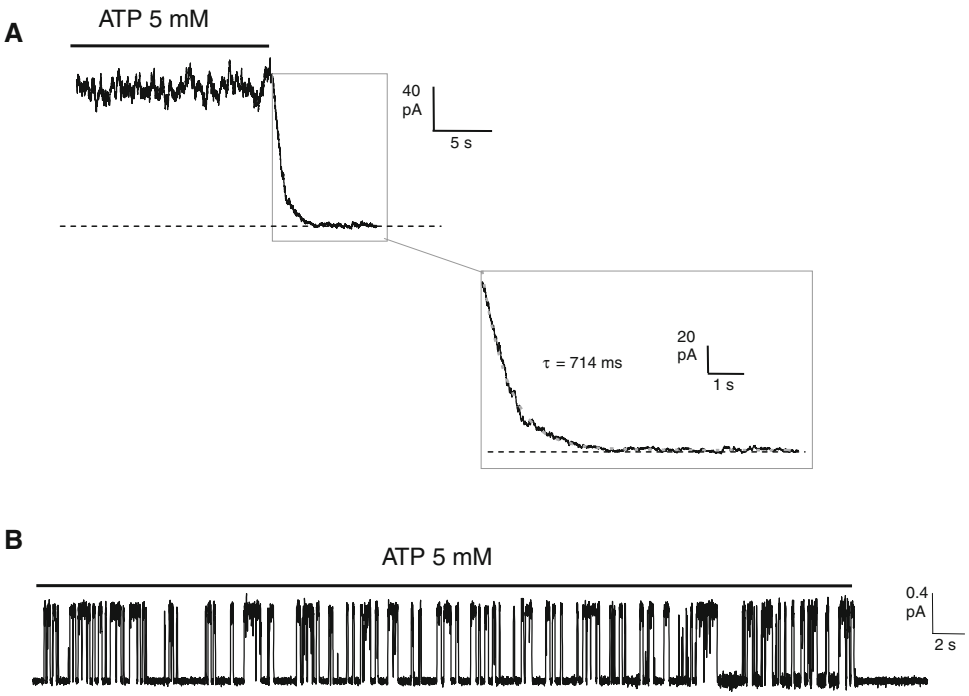

Fig. 1 Macroscopic and microscopic currents of wild-type (WT)-CFTR. **a** Macroscopic WT-CFTR current after a rapid removal of 5 mM ATP. The current relaxation was fitted with a function: $f(t) = A \cdot \exp(-t/\tau) + C$, where τ and A is time constant and amplitude of the current relaxation and C is a constant (*gray dashed line* in *inset*). Note that the constant C approximates a very small and slow component possibly underlied by long-lasting opened WT-CFTR channels (see Fig. 5). **b** A representative current trace of WT-CFTR channel showing immediate closing upon a rapid removal of ATP

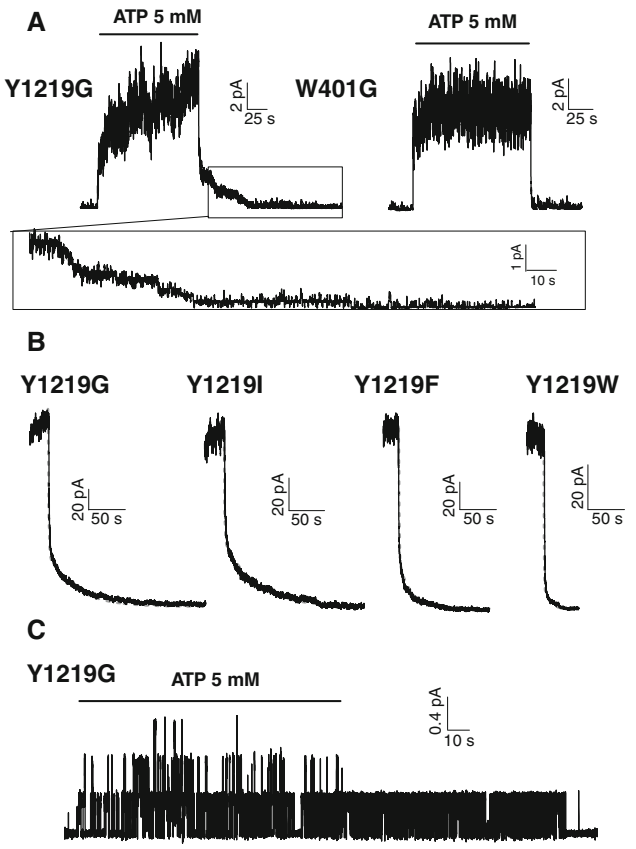

Fig. 2 Macroscopic current relaxations for Y1219 and W401 mutants. **a** Representative traces of Y1219G and W401G macroscopic currents responding to a rapid application and removal of 5 mM ATP. The extended trace marks part of the slow current decay of Y1219G-CFTR after ATP removal. Note that the current traces reveal some minor channel activities supposed to be in a minor gating modes of CFTR [8, 9]. **b** Macroscopic current relaxations upon ATP wash-out for Y1219G, Y1219I, Y1219F and Y1219W. The current relaxations were fitted with a double exponential function. The time constant and the fraction of the slow component are summarized in Fig. 3. **c** Single channel recording of Y1219G-CFTR showing a long opening even after ATP removal

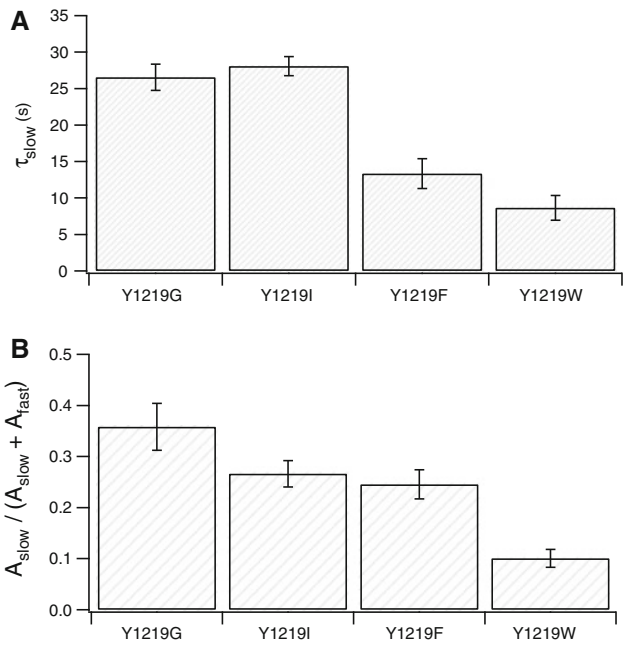

Fig. 3 Kinetic parameters of macroscopic current relaxations for Y1219G, Y1219I, Y1219F and Y1219W. **a** Time constants of the slow component of the current relaxation for the Y1219 mutants. Y1219G: $n = 5$; Y1219I: $n = 7$; Y1219F: $n = 4$; and Y1219W: $n = 5$. **b** Fraction of the slow component ($A_{slow}/A_{slow} + A_{fast}$) for the Y1219 mutants. Y1219G: $n = 5$; Y1219I: $n = 7$; Y1219F: $n = 4$; and Y1219W: $n = 5$

somewhere between Y1219W and Y1219I. It should be noted that these long-lasting events also occur in patches containing only a number of channels with discernable opening and closing steps (Fig. 2c). The time constants of the initial fast phase for all the Y1219 mutants are within hundreds of milliseconds (τ_{fast}: 504 ± 76 ms, $n = 5$ for Y1219G; 450 ± 44 ms, $n = 7$ for Y1219I; 571 ± 142 ms, $n = 4$ for Y1219F; 513 ± 66 ms, $n = 5$ for Y1219W) and there is not significant difference in the fast time constant among these Y1219 mutants (Fig. 2b).

On the other hand, the time constants of the slow component for Y1219F and Y1219W are shorter than those of Y1219G and Y1219I (Fig. 3a). More importantly, the fraction of the slow component (Fig. 3b) shows a gradual decrease as the mutation becomes less 'preserved' (Y1219G > Y1219I > Y1219F > Y1219W). It should be noted that, for technical reasons (detailed in "Discussion"), our calculation of the fraction of the slow component is somewhat overestimated for Y1219F and Y1219W. The actual values for Y1219F and Y1219W are lower than those shown in Fig. 3b. Nevertheless, the rank order for the fraction of the slow component shown in Fig. 3b correlates well with the order of changes in ATP affinity reported previously for these Y1219 mutants [21].

A high binding affinity ATP analogue P-ATP diminished long-lasting openings induced by Y1219 mutation

The experimental data with the Y1219 mutations (Figs. 2, 3) suggest that the instability of ATP binding at NBD2 might lead to the long-lasting openings of CFTR channels. If indeed weakening of ATP binding by the Y1219 mutations is responsible, one may expect that a high affinity ATP analog could affect the appearance of these long-lasting openings. We have previously characterized several high-affinity hydrolysable ATP analogs and found that N^6-(2-phenylethyl)-ATP (or P-ATP) has an apparent affinity 50-fold higher than ATP for WT-CFTR [11]. The high affinity of P-ATP was suggested to come from its slow dissociation rate from NBD1 and NBD2 whereas the closing rate in P-ATP was just a little slower than that in

ATP because of their similar rate-limiting hydrolyzing rates [11]. Figure 4 shows the effects of application and removal of 5 mM ATP and 100 μM P-ATP on the macroscopic current of the Y1219I mutant. In the same patch, the application of 100 μM P-ATP elicited a macroscopic current, which decayed monotonically upon washout of P-ATP. Subsequent application of 5 mM ATP induced a biphasic current rise. Note that the initial current elicited by ATP is roughly of the same amplitude as that induced by 100 μM P-ATP. Unlike the current in the presence of P-ATP, the current in the presence of ATP shows a slow second rising phase (Fig. 4a). Upon washout of ATP, a slow phase of current decay becomes discernable. After the current returned to the base line, the application of 100 μM P-ATP again elicited a current that decayed monotonically upon washout of the nucleotide (Fig. 4a).

Since the slow phase of current decay in Y1219I-CFTR channels was diminished when opened by P-ATP (Fig. 4a), we next examined the accessibility of P-ATP to the binding site during the long-lasting open state by testing if P-ATP can rapidly close Y1219I channels in the long-lasting open state. We first opened Y1219I-CFTR channels with ATP. Upon removal of ATP, we applied P-ATP briefly (~3 s) at the beginning of the slow phase of current relaxation. Interestingly, this brief application of P-ATP, although eliciting a macroscopic current with a rapid decay upon P-ATP removal, did not alter the time course of the slow current relaxation (Fig. 4b). These results suggest that, once the channel is in this stable open state, P-ATP cannot access its binding site.

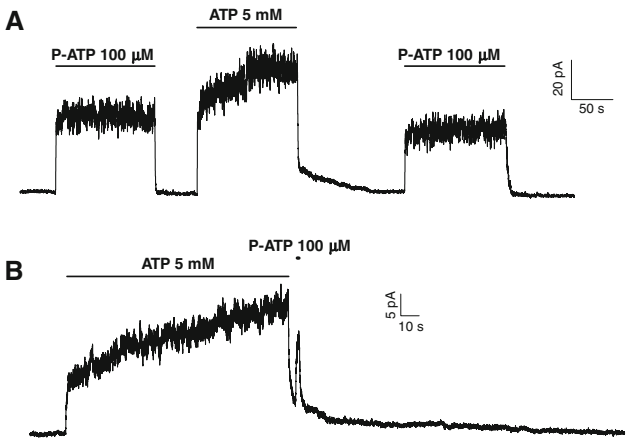

Fig. 4 Effects of P-ATP on Y1219I mutant. **a** A representative macroscopic current trace of Y1219I-CFTR responding to rapid application and removal of 5 mM ATP or 100 μM P-ATP. Similar observations were made in nine membrane patches. **b** Effects of a brief rapid application of P-ATP on Y1219I macroscopic current during the slow phase of the current relaxation after ATP wash-out. A representative current trace from five similar experimental results is shown

Long-lasting openings could be observed rarely in WT-CFTR

As shown in Fig. 1a, for WT-CFTR, removal of ATP in excised inside-out patches resulted in a fast current decay with a time constant of ~650 ms. However, occasionally, we also observed some long-lasting opening events that do not fit in the single exponential decay of the current relaxation (Fig. 5a; enlarged inset). Because this slow component is only a minor fraction of the overall current relaxation, it is technically difficult to estimate the time constant accurately. However, by eye inspection, the relaxation time constant for this component should be at least on the order of tens of seconds. With an assumption that the open probability is 0.4 and 1 in the normal and the long-lasting opening modes, respectively, and that the single channel amplitude was 0.4 pA, it was roughly estimated that about 20 channels (1.4%) of all 1,340 WT-CFTR channels in five patches were in the long-lasting opening state. This means that the contribution of the channels in the long-lasting opening state to the macroscopic current should be around 2% of the total.

At the single channel level, it was extremely rare but certain that long-lasting openings of WT-CFTR also exist in patches containing just a few channels (Fig. 5b, upper panel). Thus, these data suggest that WT-CFTR channels can assume another minor open state in addition to the major one with a time constant around hundreds of milliseconds as reported previously [e.g., 12, 20]. Also entering into the long-lasting opening state seemed not to introduce a significant change in the single channel conductance (Fig. 5b, lower panel).

Since the life time of these long-lasting openings (in tens of seconds) is very similar to that of hydrolysis-deficient CFTR mutants such as K1250A and E1371S [9, 12, 17–20], it seems difficult to explain these events with the conventional theory that ATP hydrolysis closes the channel. Based on the experimental findings that mutations that lower the binding affinity of ATP at NBD2, but not at NBD1, will promote this stable open state, one possible explanation is that these events represent an open state where ATP is irreversibly located in a wrong position for hydrolysis or has dissociated from its NBD2 binding pocket.

Discussion

Here, we report that mutations that decrease ATP binding affinity at NBD2 of CFTR significantly increase the prevalence of the long-lasting opening events with a time constant of tens of seconds. We show a correlation between the occurrence of these events and the degree of

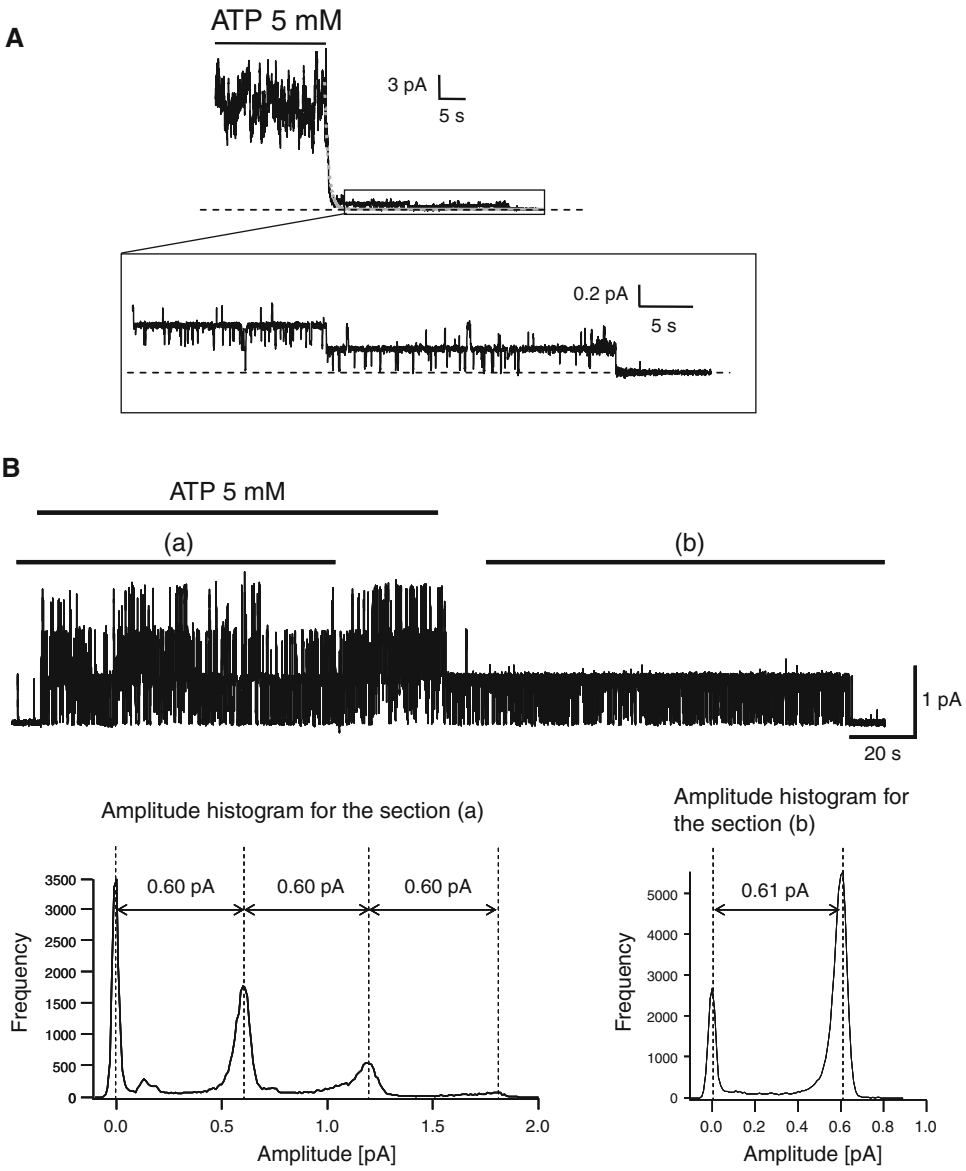

Fig. 5 Long-lasting openings observed in macroscopic and microscopic currents of wild type (WT)-CFTR. **a** A representative of WT-CFTR showing long-lasting openings after a rapid removal of 5 mM ATP. **b** Single channel current recordings obtained from a patch containing three active WT-CFTR channels (*upper panel*). One of the channels remains open for more than 1 min after the removal of ATP whereas the other two channels close within 1 s (*upper panel*). The *lower panel* shows amplitude histograms from the *sections* (*a*) and (*b*) in the single channel current data shown in the *upper panel*. All the three WT-CFTR channels in normal gating mode showed the almost same single channel conductance of 0.60 pA [*section* (*a*)]. Then one of the three channels went into the long-lasting opening state and its single channel conductance was 0.61 pA [*section* (*b*)]. Although we cannot know which of the three channels went into the long-lasting opening state, the single channel conductance should not be significantly changed because all three channels have the same single channel conductance of 0.60 pA, anyway

alternations in ATP binding affinity at NBD2 induced by different mutations at the Y1219 residue. We also describe that WT channels rarely show this extremely stable open state. We will discuss the biophysical and structural significance of these novel observations.

Instability of ATP binding to NBD2 leads to a stable open state

ATP-dependent gating of CFTR is unique in that the ligand ATP is hydrolyzed and this ATP breakdown provides the driving force for channel closing [7]. Because of a rapid hydrolysis rate, WT-CFTR channels normally close at a rate of $\sim 2\ s^{-1}$. On the other hand, CFTR channels can open for hundreds of seconds when ATP hydrolysis is abolished by mutations such as E1371S or K1250A [9, 12, 17–20].

Although the crystal structure of CFTR's NBD2 has yet to be published, we have provided functional data supporting the idea that Y1219 residue interacts with the adenine ring of bound ATP at NBD2 [21], an equivalent role of W401 at NBD1 [16]. In this study, we found that mutations of Y1219 increase the probability of the long-lasting openings, which were observed as a slow component in the macroscopic current relaxation after a rapid removal of ATP. In contrast, decreasing ATP binding in NBD1 by the W401G mutation does not induce a significant slow component of current relaxations (Fig. 2a). On the other hand, the time constants for the fast component in different Y1219 mutations are not significantly different

from that of WT-CFTR. Since the closing rate of CFTR channel is thought to be determined by the rate of ATP hydrolysis at NBD2 [13], these results suggest that, most of the time, the Y1219 mutant can assume a normal rate of ATP hydrolysis at NBD2.

We also observed an interesting correlation between the probability of the stable open state and the degree of alterations of the ATP binding affinity induced by different Y1219 mutations. Although the results with Y1219G and Y1219I mutations are quantified accurately, this may not be the case with the data for Y1219F and Y1219W. First, like WT-CFTR, the slow component for Y1219W is too obscure for reliable curve fitting. Second, the existence of the slow gating mode [8] may affect the value of the relaxation time constant. This effect is expected to be small when the probability of the stable open state is high such as in Y1219G or Y1219I. Some dephosphorylation might affect the current relaxation which, while it should not be significant [9], should also be noted. Thus, the differences in the fraction of the slow component among Y1219 mutations are somewhat underestimated.

Nevertheless, the relationship between ligand binding stability and the probability of the stable state is further supported by our results with P-ATP (Fig. 4). Since P-ATP has a >50-fold higher affinity to NBD2 compared with ATP [11], P-ATP may also assume a high affinity for the Y1219 mutants. Indeed, while we need millimolar ATP to elicit significant currents from Y1219I-CFTR, 100 μM P-ATP can generate a current similar to the initial current induced by 5 mM ATP (Fig. 4a), indicating that P-ATP is more potent in opening Y1219I channels. Thus, the high-affinity ATP analog, P-ATP, can effectively abolish the stable open state of Y1219I-CFTR, corroborating the idea that weakening ATP binding at NBD2 promotes the stable open state.

Structural/molecular mechanisms underlying the long open state

While the definitive mechanism for the long-lasting openings of Y1219-mutated CFTR channels is still unknown, based on the "instability of ATP binding" concept, we consider two scenarios for the destiny of the bound ATP at NBD2 after the channel is opened through NBD dimerization.

First, the bound ATP dissociates from the ATP Binding Pocket 2 (ABP2) formed by the Walker A and B motifs of NBD2 and the signature sequence of NBD1 [3, 7]. Since the ABP2 is now empty, the channel closes not by ATP hydrolysis but through a nonhydrolytic closing similar to that of hydrolysis-deficient mutants (kiss and run hypothesis; see Fig. 6a). Second, the bound ATP, due to a loosened binding by mutations, is dislocated from the optimal position for ATP hydrolysis. Thus, before the dislocated ATP returns to the original position, the channel remains open (stay and stray hypothesis; see Fig. 6b). Although P-ATP is able to shorten the lifetime of ATP-opened Y1219I mutants (Fig. 4a), we could not conclude whether the bound ATP has dissociated so that the binding pocket is now unoccupied or the dislocated ATP is still in the binding pocket. The negative result shown in Fig. 4b also cannot rule out the possibility that the binding pocket is indeed vacant but becomes inaccessible to P-ATP.

The "kiss and run" hypothesis (Fig. 6a) could explain the relationship between the fractions of the slow component and the extent of alterations in ATP affinity for

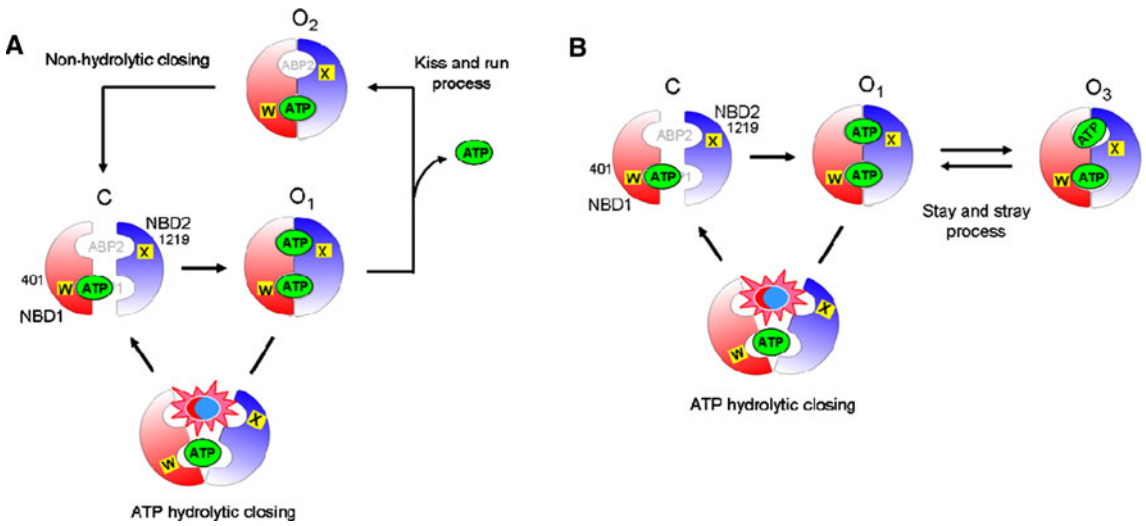

Fig. 6 Schematic presentation of **a** "kiss and run" hypothesis and **b** "stay and stray" hypothesis for the Y1219 mutation-induced long-lasting openings in CFTR channels

different Y1219 mutations as shown in Figs. 2b and 3b. Since, once the channel is opened by ATP, the bound ATP is sandwiched at the dimer interface, a dissociation of bound ATP, if it happens, is expected to be extremely slow. Indeed, for WT-CFTR, these long-lasting openings are rarely seen. In fact, even when ATP affinity is drastically reduced by mutations such as Y1219G and Y1219I, the probability of the stable open state remains quite low compared to the short-lived open state. This is expected since the binding affinity of ATP in the open state (i.e., in NBD dimer) should be much higher and less sensitive to mutations than that for the closed state (NBD in a monomeric configuration). However, the simple "kiss and run" hypothesis could not explain the mutation-dependent slow time constants (Fig. 3a) because all the Y1219 mutants close through a common non-hydrolytic mechanism (Table 1). Without ATP hydrolysis, the slow time constants should be similar among all the Y1219 mutants (Fig. 7a).

Table 1 Parameter sets (s^{-1}) for the "kiss and run" hypothesis

	k_{CO1}	k_{O1C}	k_{O1O2}	k_{O2C}
Y1219G	3 or 0[a]	3	2×10^{-2}	3.8×10^{-2}
Y1219I	↑	↑	1.3×10^{-2}	↑
Y1219F	↑	↑	1.1×10^{-2}	↑
Y1219W	↑	↑	4×10^{-3}	↑

[a] k_{CO1} was set to 3 s^{-1} for simulating the "with ATP" condition and 0 s^{-1} for simulating the "after the ATP washout" condition. Note that we employed the common values for k_{CO1} in all the Y1219 mutants whereas they should actually have different k_{CO1} values [21], because it does not affect the reproduced current relaxation

For an alternative hypothesis, the channel is closed by ATP hydrolysis, but, because of the mutation, ATP wanders inside the ABP2 for some time before it settles in a proper position for optimal hydrolysis. This hypothesis may be able to explain the mutation-dependence of the time constant (Fig. 3a). The less conserved the mutation, the more room for ATP to wander (thus the more time it takes) before it can be hydrolyzed. However, this hypothesis predicts a single slow exponential current relaxation because drifting of ATP away from the optimal position simply delays channel closing by slowing down the hydrolysis rate. This is clearly not the case (see Fig. 2a, b).

To explain the double exponential current relaxation, one has to assume a discrete state when ATP is in a dislocated position. In the "stay and stray" hypothesis (Fig. 6b), a slow entry into this state O_2 and a slow exit from O_2 state in effect confer two open states (thus two time constants). If different mutations at Y1219 can affect the entry rate and the exit rate differently as shown in Table 2, this hypothesis may explain most of the results

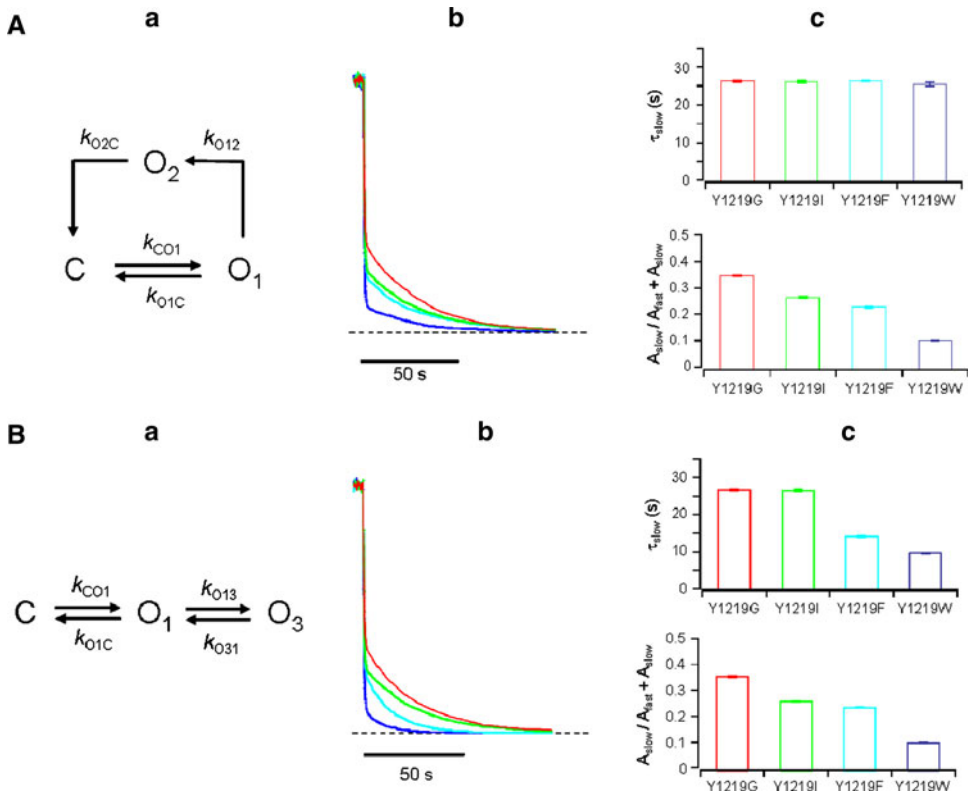

Fig. 7 Comparison of **a** "kiss and run" hypothesis and **b** "stay and stray" hypothesis using a Monte Carlo computer simulation. **a** Gating kinetic model for each hypothesis. Kinetic parameters are shown in Tables 1 and 2, respectively. **b** Representative reproduced macroscopic current relaxations mimicking those in Y1219G (*red*), Y1219I (*green*), Y1219F (*cyan*) and Y1219W (*blue*). **c** Summary of the kinetics of the reproduced current relaxation. Mean ± SEM obtained from 10 reproduced current relaxations with different random number seeds are shown

Table 2 Parameter sets (s^{-1}) for the "stay and stray" hypothesis

	k_{CO1}	k_{O1C}	k_{O1O3}	k_{O3O1}
Y1219G	3 or 0[a]	3	2×10^{-2}	3.8×10^{-2}
Y1219I	↑	↑	1.3×10^{-2}	3.8×10^{-2}
Y1219F	↑	↑	2×10^{-2}	7×10^{-2}
Y1219W	↑	↑	1×10^{-2}	1×10^{-1}

[a] k_{CO1} was set to 3 s^{-1} for simulating the "with ATP" condition and 0 s^{-1} for simulating the "after the ATP washout" condition. Note that we employed the common values for k_{CO1} in all the Y1219 mutants whereas they should actually have different k_{CO1} values [21], because it does not affect the reproduced current relaxation

shown in Fig. 3 (especially the graded changes in both the time constant and the fraction of the slow phase) (Fig. 7b).

It should be noted that this scenario also depicts that the ligand is "dissociated" from its original binding site. The only difference between this proposition and the kiss and run hypothesis is whether ATP actually detaches from the protein. However, it seems difficult to envision two discrete spaces for ATP since the binding pocket is tightly sandwiched at the NBD dimer interface. In addition, the slow time constant for Y1219G is not different from that for Y1219I despite the fraction of the slow phase is different between these two mutants. Perhaps an integration of both hypotheses is necessary to explain all the observations.

Long-lasting openings of WT-CFTR

The long-lasting openings of WT-CFTR observed in the current study (Fig. 5) significantly deviate from the usual behavior reported in the literature for WT channels. While we made these observations in a mammalian expression system, similar stable open states of WT channels are observed in membrane patches excised from *Xenopus* oocytes injected with WT-CFTR cRNA (Dr. Laszlo Csanady, personal communication). It has been reported that WT-CFTR exhibits at least three different gating modes [8]. These include the slow gating mode, the fast gating mode and a *high-Po* mode. Although this mode shift behavior was characterized initially with a CFTR construct whose regulatory domain was removed (ΔR-CFTR), the slow and fast gating modes are regularly observed in normal WT-CFTR [8]. The long-lasting openings shown in the present study could represent the rarely observed *high-Po* mode reported for the ΔR-CFTR [8].

Conclusions

In this paper, we conclude that a stable binding of ATP to NBD2 is required forthe normal, fast gating cycle of CFTR channel, and that instability of ATP binding frequently halts the CFTR gating cycle in the open state presumably through a slowdown of ATP hydrolysis at NBD2. It is worth noting that other members of ABC superfamily, e.g., *p*-glycoprotein and multi-drug resistance proteins, share the same NBD mechanism underlying their ATP-dependent active drug transport. Our results suggest that destabilizing ATP binding could halt the function of this class of proteins by effectively trapping the transporter in a stable state.

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References

1. Riordan JR, Rommens JM, Kerem BS, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1072
2. Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell 68:809–818
3. Vergani P, Lockless SW, Nairn AC, Gadsby DC (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. Nature 433:876–880
4. Locher KP, Lee AT, Rees DC (2002) The *E coli* BtuCF structure: a framework for ABC transporter architecture and mechanism. Science 296:1091–1098
5. Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, Hunt JF (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimmer. Mol Cell 10:139–149
6. Chen J, Lu G, Lin J, Davidson AL, Quiocho A (2003) A tweezer-like motion of the ATP binding cassette dimer in an ABD transport cycle. Mol Cell 12:651–661
7. Chen TY, Hwang TC (2008) CLC-0 and CFTR: chloride channels evolved from transporters. Physiol Rev 88:351–387
8. Bompadre SG, Ai T, Cho JH, Wang X, Sohma Y, Li M, Hwang TC (2005) CFTR gating I: characterization of the ATP-dependent gating of a phosphorylation-independent CFTR construct (ΔR-CFTR). J Gen Physiol 125:361–375
9. Bompadre SG, Cho JH, Wang X, Zou X, Sohma Y, Li M, Hwang TC (2005) CFTR gating II: effects of nucleotide binding on the stability of open states. J Gen Physiol 125:377–394
10. Powe AC, Al-Nakkash L, Li M, Hwang TC (2002) Mutation of Walker-A lysine 464 in cystic fibrosis transmembrane conductance regulator reveals functional interaction between its nucleotide-binding domains. J Physiol 539:333–346
11. Zhou Z, Wang X, Li M, Sohma Y, Zou X, Hwang TC (2005) High affinity ATP/ADP analogues as new tools to study CFTR gating. J Physiol 569:447–457
12. Vergani P, Nairn AC, Gadsby DC (2003) On the mechanism of MgATP-dependent gating of CFTR Cl-Channels. J Gen Physiol 121:17–36
13. Gadsby DC, Vergani P, Csanady L (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. Nature 440:477–483
14. Aleksandrov L, Aleksandrov AA, Chang XB, Riordan JR (2002) The first nucleotide binding domain of cystic fibrosis transmembrane conductance regulator is a site of stable nucleotide

interaction, whereas the second is a site of rapid turnover. J Biol Chem 277:15419–15425
15. Basso C, Vergani P, Nairn AC, Gadsby DC (2003) Prolonged nonhydrolytic interaction of nucleotide with CFTR's NH2-terminal nucleotide binding domain and its role in channel gating. J Gen Physiol 122:333–348
16. Lewis HA, Buchanan SG, Burley SK, Conners K, Dickey M, Dorwart M, Flower R, Gao X, Guggino WB, Hendrickson WA et al (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. EMBO J 23:282–293
17. Aleksandrov AA, Chang XB, Aleksandrov L, Riordan JR (2000) The non-hydrolytic pathway of cystic fibrosis transmembrane conductance regulator ion channel gating. J Physiol 528:259–265
18. Carson MR, Travis SM, Welsh MJ (1995) The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. J Biol Chem 270:1711–1717
19. Gunderson KL, Kopito RR (1995) Conformational states of CFTR associated with channel gating: the role ATP binding and hydrolysis. Cell 82:231–239
20. Zeltwanger S, Wang F, Wang GT, Gillis KD, Hwang TC (1999) Gating of cystic fibrosis transmembrane conductance regulator chloride channels by adenosine triphosphate hydrolysis quantitative analysis of a cyclic gating scheme. J Gen Physiol 113:541–554
21. Zhou Z, Wang X, Liu HY, Zou X, Li M, Hwang TC (2006) The two ATP binding sites of cystic fibrosis transmembrane conductance regulator (CFTR) play distinct roles in gating kinetics and energetics. J Gen Physiol 128:413–422
22. Cortic T, Zhang P, Todorovic N, Canessa CM (2003) The extracellular domain determines the kinetics of desensitization in acid-sensitive ion channel 1. J Biol Chem 278:45240–45247