Converting Nonhydrolyzable Nucleotides to Strong Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Agonists by Gain of Function (GOF) Mutations*§

George Okeyo†1, Wei Wang†1, Shipeng Wei†1, and Kevin L. Kirk*52

From the Departments of†1 Cell, Developmental, and Integrative Biology and §5 Neurobiology, Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

Background: ATP-gated CFTR channels are weakly activated by nonhydrolyzable nucleotides.

Results: GOF mutations promote ATP-free CFTR activity, proportionately increase activation by nonhydrolyzable nucleotides, and rescue cystic fibrosis gating mutants.

Conclusion: Nonhydrolyzable nucleotides are partial CFTR agonists that are converted to stronger agonists by GOF mutations like partial agonists of conventional ligand-gated channels.

Significance: Defective CFTR gating can be rescued by bypassing ATP binding/hydrolysis.

Cystic fibrosis transmembrane conductance regulator (CFTR) is the only ligand-gated ion channel that hydrolyzes its agonist, ATP. CFTR gating has been argued to be tightly coupled to its enzymatic activity, but channels do open occasionally in the absence of ATP and are reversibly activated (albeit weakly) by nonhydrolyzable nucleotides. Why the latter only weakly activates CFTR is not understood. Here we show that CFTR activation by adenosine 5′-(thiotriphosphate) (ATPγS), adenosine 5′-(β,γ-imino)triphosphate (AMP-PNP), and guanosine 5′-O-(thio)triphosphate (GTPγS) is enhanced substantially by gain of function (GOF) mutations in the cytosolic loops that increase unliganded activity. This enhancement correlated with the base-line nucleotide-independent activity for several GOF mutations. AMP-PNP or ATPγS activation required both nucleotide binding domains (NBDs) and was disrupted by a cystic fibrosis mutation in NBD1 (G551D). GOF mutant channels deactivated very slowly upon AMP-PNP or ATPγS removal (τdeac ~ 100 s) implying tight binding between the two NBDs. Despite this apparently tight binding, neither AMP-PNP nor ATPγS activated even the strongest GOF mutant as strongly as ATP. ATPγS-activated wild type channels deactivated more rapidly, indicating that GOF mutations in the cytosolic loops reciprocally/allosterically affect nucleotide occupancy of the NBDs. A GOF mutation substantially rescued defective ATP-dependent gating of G1349D-CFTR, a cystic fibrosis NBD2 signature sequence mutant. Interestingly, the G1349D mutation strongly disrupted activation by AMP-PNP but not by ATPγS, indicating that these analogs interact differently with the NBDs. We conclude that poorly hydrolyzable nucleotides are less effective than ATP at opening CFTR channels even when they bind tightly to the NBDs but are converted to stronger agonists by GOF mutations.

The ATP-gated anion channel that is encoded by the cystic fibrosis gene (CFTR) has two unique features: (i) it is the only member of the ABC transporter superfamily that is known to function as an ion channel, and (ii) it is the only ligand-gated ion channel that hydrolyzes its ligand. Like other ABC transporters, CFTR possesses an ATPase activity, but this enzymatic activity is used to regulate channel activity instead of active substrate transport (for reviewed, see Ref. 1). CFTR binds two ATP molecules at the interface of a tight dimer of nucleotide binding domains (NBDs) to promote channel opening (2). Hydrolysis of one of the ATPs (at site 2; site 1 is catalytically inactive) has been argued to speed channel closing by destabilizing the NBD dimer (3–5). CFTR channels can close in the absence of ATP hydrolysis but on a much slower time scale such that most closings follow hydrolysis (6). On this basis CFTR channel gating has been proposed to be “strictly coupled” to its catalytic activity (6). CFTR gating is further regulated by phosphorylation of a unique R domain by cyclic nucleotide-dependent protein kinases, which enhances channel opening by unknown mechanisms (7, 8).

The concept that CFTR channel closing is tightly coupled to its enzymatic activity is a good first approximation for highly phosphorylated channels with ATP as the ligand. This view is supported by the observation that mutations that disrupt ATP hydrolysis at site 2 cause long open channel bursts of phosphorylated CFTR channels that last many seconds to minutes and that deactivate with correspondingly slow time courses upon ATP removal (6, 9, 10). CFTR channels are (almost) always

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† These authors contributed equally to this study.

‡ To whom correspondence should be addressed: Dept. of Cell, Developmental, and Integrative Biology, 9828 MCLM, University of Alabama at Birmingham, 1720 2nd Ave. South, Birmingham, AL 35294-0005. Tel.: 205-934-3122; E-mail: klkirk@uab.edu.

§ The abbreviations used are: CFTR, cystic fibrosis (CF) transmembrane conductance regulator; ATPγS, adenosine 5′-O-(thiotriphosphate); AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; GTPγS, guanosine 5′-3-O-(thio)triphosphate; nAChR, nicotinic acetylcholine receptor; GOF, gain of function; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid; MOT, mean open time; NBD, nucleotide binding domain.
open during these long bursts of activity, although very brief closings can be detected at high sampling frequencies (6, 10). On the other hand, the strict coupling concept does not explain the fact that CFTR channels do open occasionally in the absence of ATP (10–13) or can be reversibly activated (albeit weakly) by poorly hydrolyzable or nonhydrolyzable nucleotides such as ATPγS and AMP-PNP (14, 15). Why ATPγS and AMP-PNP only weakly activate CFTR is not well understood.

The fact that channels can open in the absence of ATP or be activated to a small degree by ATPγS and AMP-PNP indicate that CFTR gating shares features with conventional ligand-gated channels that reversibly bind rather than consume their ligands. The gating of a conventional ligand-gated channel like the nicotinic acetylcholine receptor (nAChR) is well described by an allosteric scheme in which the open state(s) is accessible in the absence of ligand (16–19). Ligand binding increases the probability of channel opening but is not absolutely required to open the channel. Channels open in the absence of ligand (usually infrequently), and channels close even when bound to the ligand. Ligand binding biases the equilibrium toward the open state; unbinding biases the equilibrium toward the closed state (16, 18, 20). The cyclic nature of these schemes demands that the open and closed states have different ligand affinities; i.e. higher for open channels. Isomerization or gain of function (GOF) mutations that increase unliganded activity have been described for typical ligand-gated channels (16, 19, 21). By definition, these GOF mutations increase ligand-free channel activity and proportionately increase the efficacies of partial agonists that bind to the channel but normally are less effective than the native ligand (full agonist) at promoting channel opening. GOF mutations also increase ligand sensitivity or occupancy because they shift the equilibrium toward the higher affinity open state, a phenomenon termed reciprocity (18–22). Thus, GOF or isomerization mutations promote activation by partial agonists in two related ways: (i) by increasing their binding to the channel at normally sub-saturating concentrations and (ii) by increasing the efficacies of these agonists to promote opening when they are bound to the channel.

CFTR channels also open in the absence of their ligand ATP as mentioned above (10–13). Indeed, truncated CFTR channels that lack one of the two NBDs open occasionally and independently of ATP (13, 23, 24). Furthermore, several classes of GOF mutations that promote ATP-free CFTR activity have been reported (13, 25, 26), at least one of which reciprocally enhances the ATP sensitivity of channel gating (13). These recent findings support the view that CFTR gating obeys many of the allosteric principles that describe the gating of conventional ligand-gated channels. Here we further tested the applicability of this conceptual framework to CFTR by examining the activation of wild type and mutant channels by a series of poorly hydrolyzable nucleotides that normally are weak CFTR channel activators (AMP-PNP, GTPγS, and ATPγS; Refs. 14 and 15). We reasoned that if these analogs are partial agonists in the nomenclature of classic allosteric activation schemes (16–21) then they should be converted to stronger agonists by GOF mutations that increase ligand-free activity. Our results support this prediction, provide insights into why these analogs normally are weak CFTR activators, and show that GOF mutations can rescue the defective gating of CF channels with mutations in the NBD signature sequences.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Constructs, and Transfections—HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1 mM penicillin-streptomycin (Invitrogen). Transient transfection of HEK 293T cells with wild type (WT) or mutant CFTR cDNA was performed using the Lipofectamine 2000 transfection kit (Invitrogen) following the manufacturer’s recommendations. The QuikChange site-directed mutagenesis kit (Agilent Technologies) was used to generate point mutations and deletion mutations using appropriate mutagenic oligonucleotides. All mutants were verified by DNA sequencing and subcloned into the pCDNA3 mammalian expression vector (Invitrogen). After transfection, cells were grown for 24–48 h at 37 °C in DMEM plus 10% FBS without antibiotics.

Electrophysiology and Data Analysis—Macroscopic and unitary currents were recorded in the excised, inside-out configuration using standard patch clamp techniques. Patch pipettes were pulled from Corning 8161 glass to tip resistance of 1.5–3.5 megaohms for macroscopic recordings and 7–12 megaohms for unitary current recordings. Pipette and bath solutions were identical and contained 140 mM N-methyl-d-glucamine, 3 mM MgCl₂, 1 mM EGTA, and 10 mM TES adjusted to pH 7.3 with HCl. Stock solutions of ATP, ATPγS, and AMP-PNP were prepared in water. The stocks were adjusted to neutral pH with NaOH before most experiments were performed using high final concentrations of nucleotide (>1.5 mM).

Macroscopic currents were recorded using a ramp protocol from +80 to −80 mV with a 10-s time period. Patches were held at −60 mV for unitary current recordings. All patch clamp experiments were performed at 21–23 °C. Macroscopic current signals were analog-filtered at 20 Hz. Unitary current signals were analog-filtered at 110 Hz and then digitally filtered at 50 Hz with Clampfit 10 software (Axon Instruments). Estimates of single channel open probabilities (Pₚ) and channel opening rates (openings/s-channel) were generated from records containing fewer than three detectable channels using Clampfit 10. Signal durations briefer than 50 ms were ignored to minimize the contribution of flickery closings due to pore block (e.g. by the pH buffer). Mean open times (MOTs, in seconds) were estimated for the same filtered multichannel records using the cycle time method described by Mathews et al. (8), where T is the length of the channel record (in seconds).

\[
\text{MOT} = \frac{(N_p)T}{\text{number of openings}} \quad (\text{Eq. 1})
\]

This method can be used to roughly estimate MOTs for multichannel patches with the assumption that there exists only one population of openings (8). Open-time histograms also were generated by pooling openings from segments of records in which only one channel had opened. The data in Fig. 2 show that the MOTs estimated for multichannel patches using the cycle time method fall within the range of open times observed in these histograms (compare Fig. 2, B and C).
**CFTR Activation by Nonhydrolyzable Nucleotides**

Fig. 3D Curve-fitting—The asymptotic equation that was used for curve-fitting in Fig. 3D was derived assuming two constant parameters: (i) a constant ratio (α) of the equilibrium constants for channel opening and closing (K_{eq}) in the presence of AMP-PNP (AMP) and in the absence of nucleotide (nuc) across all channel constructs (where α = K_{eqAMP}/K_{eqnuc} and K_{eq} = #open channels/# closed channels = P_{o}/1 – P_{o}) and (ii) a constant P_{o} in the presence of 1.5 mM ATP (P_{oATP}) across all constructs. The first assumption holds for purely constitutive mutations that solely affect channel isomerization (i.e. the closed to open transition) without directly impacting the ligand binding domains or other regulatory inputs (termed isomerization mutations by Changeux and co-workers (17, 18)); see also Ref. 16). The second assumption does not strictly hold for the tested CFTR constructs (e.g. see 13), but the error is apparently buried in the scatter in Fig. 3D.

Set α = K_{eqAMP}/K_{eqnuc} (Eq. 2)

\[ α = (P_{oAMP}/1 – P_{AMP})/(P_{oATP}/1 – P_{oATP}) \] (Eq. 3)

Rearranging and solving for P_{oAMP} gives

\[ P_{oAMP} = α/[(α – 1) + (1/P_{oATP})] \] (Eq. 4)

The data plotted in Fig. 3D are normalized P_{o} values of the form

\[ Y = 100(P_{oAMP}/P_{oATP}) \] (Eq. 5)

and

\[ X = 100(P_{oATP}/P_{oATP}) \] (Eq. 6)

Solving for Y from X using Equation 4 gives

\[ Y = 100α/[P_{oATP}(α – 1) + (100/α)] \] (Eq. 7)

The data of Fig. 3D were fit to Equation 7 assuming constant α and P_{oATP} across all constructs as stated above. Nonlinear curve-fitting was successful performed in >100 iterations using Origin 8.5 with the following output parameters for the best fit: α = 13.0 ± 2.1 (S.E.), P_{oATP} = 1.0 ± 0.1, adjusted R^2 = 0.78.

**RESULTS**

A GOF Mutation Increases CFTR Activation by Poorly Hydrolyzable Nucleotides—Fig. 1 shows the strong activation of a previously characterized GOF mutant (K978C-CFTR) by AMP-PNP, ATPγS, and GTPγS in excised inside-out macropatches. This mutation is located in cytosolic loop 3, which lies along the axis that links the NBDs to the pore in structural models of CFTR (see supplemental Fig. S1). Previously we showed that the K978C substitution increased the single channel open probabilities (P_{o}) of phosphorylated channels in the absence of nucleotide by at least 2 orders of magnitude (13). This ligand-independent activity of K978C-CFTR is detected in excised macropatch recordings as a small current that persists after removal of bath ATP by a scavenger (hexokinase/glucose) and subsequent bath perfusion with ATP-free solution (Fig. 1A). Wild type channels (WT-CFTR) exhibit negligible ATP-independent currents at this gain (Fig. 1B). WT-CFTR currents were only modestly stimulated by adding increasing concentrations of AMP-PNP, ATPγS, or GTPγS to the bath after ATP removal (Fig. 1, B, D, and F, and supplemental Fig. S2) as reported previously by several groups (10, 14, 15). In contrast, these analogs strongly increased the currents mediated by K978C-CFTR, although not to the level of saturating ATP (Fig. 1, A, C, and F and supplemental Fig. S2). Activation by the hydrolyzable GTP also was enhanced by this GOF mutation (supplemental Fig. S2). The currents activated by AMP-PNP or ATPγS were insensitive to the ATP scavenger when re-added to eliminate contaminating ATP in the commercial nucleotide preparations (e.g. Fig. 1A).

Titration experiments revealed saturable activation of the GOF mutant by each of the analogs with the highest apparent affinity for ATPγS (Fig. 1E). The ATPγS titration data were best fit to a model with two binding sites with different affinities unlike the AMP-PNP and GTPγS data (see the legend to Fig. 1E). Fig. 1F compares the maximal activation of wild type and GOF mutant channels at saturating doses of each of the analogs where the degree of activation was normalized to the currents measured in the presence of saturating ATP. The GOF mutant was much more strongly activated by each of the three analogs, although none of the poorly hydrolyzable nucleotides activated as well as ATP.

Fig. 2 shows activation of the K978C GOF mutant by a saturating dose of ATPγS (1.5 mM) at the single channel level. As we reported earlier, this mutant has a higher P_{o} than wild type CFTR both in the presence of 1.5 mM ATP and after ATP removal (13). The mean P_{o} values before and after ATP removal observed here (0.79 and 0.07, respectively) are quite similar to those reported previously for this GOF mutant (13). ATPγS addition subsequent to ATP removal markedly increased the P_{o}, in agreement with the macroscopic current data (representative record in Fig. 2A, mean data in Fig. 2C). The mean P_{o} after ATPγS activation (0.5), although not to the level of the ATP control value, was nearly an order of magnitude greater than for wild type channels at the same ATPγS concentration (mean P_{o} of ATPγS-activated wild type channels was 0.08 ± 0.03 (S.E.; n = 4), in agreement with previous reports (Ref. 10; see the Fig. 2 legend for P_{o} values for WT-CFTR under all 3 conditions). ATPγS-activated K978C-CFTR channels opened and closed dynamically (i.e. were not “locked open”) with a broad distribution of open durations that ranged from <100 ms to occasional openings that lasted 10–20 s (Fig. 2B). The open time distributions were not appreciably different between channels that were activated by ATP versus ATPγS. In agreement with this, the MOTs estimated using a previously described cycle time protocol (8) were similar for all 3 conditions (Fig. 2C). Fig. 2C shows that the increase in P_{o} induced by ATPγS was due primarily to an increase in channel opening rate.

**GOF Mutations Increase CFTR Activation by AMP-PNP in Proportion to Their Enhancement of ATP-independent Activity—**Isomerization mutations in conventional ligand-gated channels increase the efficacies of partial agonists in proportion to their effects on unliganded channel activity (16, 22). Accordingly, we compared the activation by AMP-PNP of several previously described GOF CFTR mutants with increasing degrees of unliganded activity (K978P, K978C, and K190C/K978C; see Fig. 3). All three GOF mutants were more strongly activated by...
AMP-PNP than WT-CFTR, with the lowest and greatest relative activation observed for the weakest (K978P; Fig. 3, A, C, and D) and strongest (K190C/K978C; Fig. 3, B, C, and D) GOF mutant. The scatter plot in Fig. 3D illustrates good correlation between the effects of the GOF mutations on unliganded activity and on activation by AMP-PNP. The data in this plot are well fit to an equation that was derived assuming proportional effects of the GOF mutations on unliganded activity and AMP-PNP efficacy (see “Experimental Procedures”). These effects of the GOF mutations on CFTR gating by AMP-PNP are qualitatively similar to the effects of isomerization mutations of conventional ligand-gated channels on their activation by partial agonists (16).

AMP-PNP- and ATPγS-stimulated Channels Deactivate Very Slowly; Evidence for Tight Binding—ATP-induced wild type CFTR currents deactivate within seconds of ATP removal, as ATP is hydrolyzed at site 2 (Ref. 6; and Fig. 4F). In contrast, the K978C-CFTR currents that were activated by ATPγS deactivated very slowly upon the removal of these agonists (Fig. 4, A–D). The deactivation time courses were well

FIGURE 1. Poorly hydrolyzable nucleotides strongly activate the K978C-CFTR GOF mutant. A and B, representative excised inside-out macropatch records compare activation of K978C-CFTR (A) and WT-CFTR (B) by the indicated concentrations of AMP-PNP. Ramp protocol (±80 mV). Control currents were initially activated with 110 units/ml PKA and 1.5 mM MgATP. PKA inhibitory peptide (1.4 µg/ml PKI) was added to block further phosphorylation. Hexokinase (24 units/ml) plus 10 mM glucose (indicated as Hex) was added to scavenge ATP followed by bath perfusion with ATP-free solution. Hexokinase/glucose was re-added after activation of K978C-CFTR by AMP-PNP followed by the addition of the CFTR-specific inhibitor, CFTRinh-172 (10 µM). The lower macroscopic control current for K978C-CFTR (PKA plus ATP) in panel A is due to somewhat lower expression of this GOF mutant compared with WT-CFTR and not to lower channel activity. In fact, this mutant had a substantially greater single channel Po than WT-CFTR under control conditions and after ATP removal (Ref. 13 and Fig. 2).

C and D, representative excised inside-out macropatch records compare activation of K978C-CFTR (C) and WT-CFTR (D) by ATPγS. In panel C, ATPγS was added at the arrows to final concentrations of 12.5, 25, 50, 75, 100, 250, 500, 750, 1000, 1500, and 2000 µM. In panel D the lowest concentration tested was 25 µM ranging up to 2000 µM. E, titration curves for K978C-CFTR activation by ATPγS (squares; n = 3), AMP-PNP (triangles; n = 4), and GTPγS (circles; n = 4). Symbols are the means ± S.E. AMP-PNP and GTPγS curves are the best fits to the Hill equation (GTPγS: Kd = 250 µM, Hill coefficient = 1.1; AMP-PNP: Kd = 260 µM, Hill coefficient = 1.3). ATPγS data are fit to a two-binding site model (Kd values of 15 and 238 µM). The inset shows ATPγS activation below 100 µM. F, mean percent activation by the indicated nucleotides at maximal concentrations (1–2 mM) is compared with ATP control (post PKI; pre hexokinase current). Data are the means ± S.E. (n = 3–14). *, p < 0.05 compared with wild type; **, p < 0.01 (unpaired two tailed t test).
fit to single exponential curves (Fig. 4, A–C) with time constants on the order of 100 s for the GOF mutant. Wild type channels also deactivated fairly slowly after ATPγS removal but considerably faster than K978C-CFTR (Fig. 4, C and D). The slower deactivation of the GOF mutant is consistent with the reciprocal effects of a GOF mutation on ligand occupancy that is predicted by an allosteric activation scheme like those that describe the gating of typical ligand-gated channels (16–22). In this regard, we also observed that ATP-activated K978C-CFTR channels deactivated much slower than WT-CFTR when ATP was removed either by adding the hexokinase/glucose scavenger to the bath (13) or by bath perfusion (Fig. 4F).

The deactivation time courses for ATPγS-activated K978C-CFTR channels (>100s) were considerably longer than the mean open times observed in the single channel experiments in Fig. 2 (<5s). This disparity argues that K978C channels open and close dynamically even when ATPγS is tightly bound. To explore this point further, we tracked the gating of K978C and wild type channels after ATPγS removal in patches containing sufficiently few channels to permit detection of unitary events. K978C-CFTR channels continued to open and close up to several min after ATPγS removal (Fig. 4E). Even wild type channels opened and closed many seconds after ATPγS removal as if they too opened and closed independently of ligand binding and unbinding. The slow deactivation observed especially for K978C-CFTR channels implies that ATPγS and AMP-PNP bind tightly, presumably at the NBD dimer interface (see below). Despite this apparently tight binding, they still fail to activate CFTR as strongly as ATP (Fig. 1F). Channels that are occupied by these ligands are more often closed.

**Evidence for Binding of ATPγS and AMP-PNP at the NBD Dimer Interface**—Fig. 5 shows that both NBDs are required for activation by AMP-PNP, as would be expected if this activation involves tight binding at the NBD dimer interface. Previously we reported that truncated channels that lack NBD2 but possess a GOF mutation (K978C/Δ1198-CFTR) exhibit detectable
ATP-independent currents in excised macropatches (Fig. 5B and Ref 13). ATP had no effect on the currents mediated by this NBD2 deletion construct (13) nor did AMP-PNP in the current study (Fig. 5B). It should be noted that these truncated channels are not maximally active under the conditions of this experiment; i.e. previously we observed that K978C/H9004-CFTR channels are stimulated substantially by a compound that activates CFTR currents by an unknown mechanism (curcumin; Refs. 13 and 24; see also Fig. 5, E and F).

We also examined the effect of modifying a cysteine at the NBD dimer interface (Fig. 5, C and D) on AMP-PNP activation as another test of the role of the NBD dimer in this activation. Earlier we reported that the combination of glutathione (GSH) with diamide (a strong oxidizer) rapidly inhibits WT-CFTR channel activity apparently by modifying Cys-1344 near the ABC signature sequence lining ATP binding site 1 (27). Modifying this residue with the large glutathione tripeptide would be expected to disrupt the NBD dimer and restrict access of the reactive glutathione to Cys-1344 (27).

**CF-causing Mutations in the NBD Signature Sequences and Channel Activation by Poorly Hydrolyzable Nucleotides**—Given the apparently important role of the NBD dimer in CFTR activation by poorly hydrolyzable analogs, we next tested the effects of two CF-causing mutations in the NBD signature sequences on this activation. The most common CF gating mutation (G551D) abolishes ATP-dependent channel opening by disrupting the signature sequence in NBD1 (12). This substitution is predicted to distort the NBD dimer based on structural models of CFTR and its homology to other ABC transporters (28, 29). In an earlier study we found that GOF mutations at residue 978 (K978C or K978S) promoted a substantial ATP-independent activity for the G551D mutant that could be augmented further by curcumin (13). Here we observed that K978C/G551D-CFTR channels could not be activated by ATP (Fig. 5E) and instead were slightly but reproducibly inhibited by this nucleotide. These channels were strongly stimulated by the subsequent addition of curcumin as reported previously (13). The G551D mutation also inhibited activation by AMP-PNP, although for this nucleotide a very small but reproducible stimulation was detected (Fig. 5F).

The analogous mutation in the NBD2 signature sequence (G1349D) causes milder CF disease apparently because it incompletely disrupts channel gating. G1349D-CFTR channels retain ATP-dependent activity but with a maximal single chan-
nel $P_n$ that is about 10-fold lower than for WT-CFTR (12). As reported by others (12), we detected relatively small ATP-dependent control currents for G1349D-CFTR channels in excised macropatches. These typically small currents were greatly stimulated by the presence of curcumin and another CFTR potentiator (NPPB-AM (30)) to the bath, confirming the presence of gating-defective channels in the patch (Fig. 6, A and C). Introduction of one of the GOF mutations into this CF mutant (K978C/G1349D-CFTR) substantially rescued its activity as evidenced by much higher ATP-dependent control currents and much lower relative activation by the potentiators (Fig. 6, B and C). K978C/G1349D-CFTR channels also exhibit detectable currents in the absence of any bath nucleotide as expected (Fig. 6, D–F). Interestingly, however, both constructs were poorly responsive to AMP-PNP. The G1349D results support two con-

**FIGURE 4.** CFTR currents activated by AMP-PNP or ATPγS deactivate slowly after nucleotide removal. A, macropatch record shows slow deactivation of K978C-CFTR current after AMP-PNP washout. The inset shows single exponential fit of deactivation time course. Hex, Hexokinase. B, shown is slow deactivation of K978C-CFTR current after ATPγS washout. C, WT-CFTR currents also deactivate fairly slowly after ATPγS washout but faster than K978C-CFTR currents. Current scale was changed where indicated to better resolve the WT-CFTR deactivation time course. D, mean deactivation time constants (±S.E.) were estimated from single exponential fits of currents after ATPγS washout for WT-CFTR and K978C-CFTR (n = 6 each). *, $p < 0.05$ compared with wild type by unpaired two-tailed t test. By comparison, the mean deactivation time course for ATP-activated K978C-CFTR channels after washout of 1.5 mM ATP using the same ramp protocol was $51.0 ± 3.3$ s (n = 7). The deactivation time course for ATP-activated wild type channels after ATP removal was too rapid to resolve using this ramp protocol (i.e. WT-CFTR channels deactivate faster than the 10-s ramp period after ATP removal). E, micropatch experiments at a constant holding potential (−60 mV) show that ATPγS-activated K978C-CFTR channels (top) and WT-CFTR channels (bottom) continue to open and close many seconds after removing ATPγS by bath perfusion (initiated at the arrow). The WT-CFTR patch was obtained using a larger tip pipette to optimize detection of ATPγS-activated channels. Each experiment was performed 5 times with similar results. Time to full deactivation for K978C-CFTR channels after ATPγS removal in these micropatch experiments ranged from 19 to 246 s. F, control micropatch experiments (also at −60 mV) show more rapid deactivation of WT-CFTR channels and K978C-CFTR channels after removal of ATP by bath perfusion. These patches were obtained using smaller tip pipettes to detect unitary currents in the presence of 3 mM ATP. The slower deactivation of K978C-CFTR channels after ATP removal is consistent with the slower macroscopic deactivation time course reported previously (Ref. 13; see also “Discussion”).
Inclusions. First, the defective ATP-dependent gating of G1349D-CFTR channels can be rescued substantially by a GOF mutation located in the cytosolic loops well away from the NBDs. Second, perhaps not surprisingly, ATP/H9253S and AMP-PNP interact differently with the CFTR channel even though each behaves like a partial CFTR agonist. The P-N-P linkage in the latter may reduce its ability to accommodate to distortions in the nucleotide binding pockets and dimer interface that are induced by the G1349D mutation.

DISCUSSION

Our results support the following main conclusions. First, CFTR channel activation by normally weak ligands (e.g. AMP-PNP and ATPγS) is increased substantially by GOF mutations in the cytosolic loops well away from the NBDs. Second, perhaps not surprisingly, ATPγS and AMP-PNP interact differently with the CFTR channel even though each behaves like a partial CFTR agonist. The P-N-P linkage in the latter may reduce its ability to accommodate to distortions in the nucleotide binding pockets and dimer interface that are induced by the G1349D mutation.

The potentiating effects of the GOF mutations on CFTR activation by weak ligands correlate well with their effects on unliganded gating, as predicted by classic allosteric activation schemes for isomerization mutations that increase unliganded activity. Fourth, poorly hydrolyzable nucleotides are weaker CFTR activators than ATP even when they apparently bind tightly to the NBDs, as was the case for the K978C GOF mutant.

Why Poorly Hydrolyzable Nucleotides Normally Are Weak CFTR Activators—The weak activation of CFTR channels by the poorly hydrolyzable ATPγS and the non-hydrolyzable AMP-PNP has been recognized for nearly 20 years (15). This observation initially was misinterpreted as evidence that ATP hydrolysis is used to energize channel opening. It is now well accepted that channel opening is promoted by ATP binding at the NBD dimer interface. Hydrolysis speeds the termination of open channel bursts (1, 6). The normally weak activation of wild type CFTR by AMP-PNP and ATPγS may be due in part to weaker binding than ATP. The small WT-CFTR currents that
were activated by these analogs precluded performing quantitative titrations like we performed for the K978C GOF mutant. It is conceivable that the nucleotide binding sites of wild type channels were not saturated by these analogs even at the highest concentrations tested.

Our results also indicate that, at least for the K978C GOF mutant, these analogs are less effective than ATP at promoting channel opening at saturating concentrations when they apparently bind tightly at the NBD dimer interface. The ATPγS titration curve was best fit to a two-binding site model, implying that it bound to both nucleotide binding sites, albeit with very different affinities (see also below). Yet ATPγS did not activate the GOF channels as well as ATP even at apparently saturating concentrations (e.g. Fig. 1F). In agreement with this, ATPγS-activated K978C-CFTR channels opened and closed on a much faster time scale (i.e. they were not locked open) than they deactivated upon ligand removal. This kinetic disparity is most simply explained by dynamic opening and closing of ATPγS-occupied channels rather than by channel opening being strictly coupled to the binding of this ligand. K978C channels also were observed to open and close several minutes after removing the ATPγS (Fig. 4), which supports the view that the opening and closing of ATPγS-activated channels is not tightly coupled to ATPγS binding and unbinding (although interpreting these nonsteady-state single channel experiments is complicated by the fact that we cannot distinguish di-liganded channels from mono-liganded channels that have unbound one ligand molecule; see also below). Wild type channels also continued to open and close many seconds after removing this ligand. In our view the simplest explanation of these results is that ATPγS (and

**FIGURE 6. Rescue of G1349D-CFTR gating by K978C and differential activation of this NBD2 mutant by ATPγS and AMP-PNP.** A, a representative macropatch record shows low control current for G1349D-CFTR (1.5 mM ATP) and large activation by serial addition of 10 μM S-nitro-2-(3-phenylpropylamino)benzamide (NPPB-AM) and 30 μM curcumin. B, a corresponding macropatch record for K978C/G1349D-CFTR shows large control current and relatively small activation by NPPB-AM and curcumin. C, shown are mean control currents (1.5 mM ATP) and mean -fold activation by the combination of curcumin and NPPB-AM for G1349D-CFTR and K978C/G1349D-CFTR (n = 6 – 17; **, p < 0.01 compared with G1349D). D and E, representative macropatch records show much smaller activation of K978C/G1349D-CFTR or G1349D-CFTR current by 2 mM AMP-PNP versus 1.5 mM ATPγS. Hex, hexokinase. F, mean relative activation of G1349D-CFTR and K978C/G1349D-CFTR by AMP-PNP and ATPγS. Percent stimulation was calculated by normalizing the increase in current above base line (i.e. current in the absence of nucleotide) to the control current before ATP removal by hexokinase/glucose. **, p < 0.01 compared with AMP-PNP.
presumably AMP-PNP) is a classic partial agonist for the GOF mutants and probably for wild type CFTR as well. Like partial agonists for receptors and conventional ligand-gated channels, these nonhydrolyzable nucleotides can bind to CFTR but have reduced efficiencies for opening the channel than the full agonist, ATP.

The apparently lower efficiencies of these agonists presumably reflect differences in the conformation of the NBD dimer that regulates channel opening when CFTR is occupied by these ligands versus ATP. AMP-PNP and ATPγS apparently bind tightly at the NBD dimer interface (especially for the GOF mutants), but the resulting dimer is not as productive or as effective at promoting channel opening as when ATP is bound. The conclusion that these analogs can bind tightly at the NBD dimer interface in CFTR is supported by structural studies of other ABC transporters for which AMP-PNP binds sufficiently tightly to support the formation of an NBD dimer in the full-length transporter (e.g. MsbA (29)). But such NBD dimers are unlikely to be structurally identical to those formed in the presence of the native ligand ATP. In this regard, Moody et al. (33) reported that AMP-PNP and ATPγS were much less effective than ATP at supporting the formation of stable dimers of the isolated NBDs of the MJ0796 or MJ1267 bacterial transporter cassettes.

How GOF Loop Mutations Increase CFTR Activation by Weak Activators—The positions of the GOF mutations studied here (Lys-190 and -978) lie along the axis that links the NBDs to the pore when mapped onto the available crystal structures of homologous ABC transporters (13). Many previously described GOF or isomerization mutations for classical ligand-gated channels similarly map to the symmetry axis between the ligand binding sites and the pore (19, 21, 31). The best studied isomerization or GOF mutants are those characterized for the nAChR by Auerbach and co-workers (16, 21, 31). GOF mutations of the nAChR increase unliganded channel activity, convert partial agonists, into stronger agonists and increase ligand sensitivity by biasing the equilibrium toward the higher affinity open state. Some nAChR GOF mutations are disease-relevant, for example, mutations that cause congenital myasthenic syndrome by increasing channel activation by the normally weak agonist choline (32). The CFTR GOF mutants that we have characterized have qualitatively similar properties to those reported for nAChRs and other ligand-gated channels (19, 31, 32). These mutations increased unliganded (ATP-free) activity and ATP sensitivity, as reported earlier (13), and correspondingly increased CFTR activation by the normally weak activators AMP-PNP, ATPγS, and GTPγS (shown here). How GOF mutations impact the structures of either the nAChR or the CFTR channel to enhance unliganded activity is not yet known. We speculated earlier that such mutations may increase TM flexibility to permit more frequent CFTR channel opening in the absence of ligand binding, which we conceptualized in an elastic spring model (13). In this regard, the substitutions at position Lys-978 that had the strongest GOF effects (K978C, K978S, and K978P; Ref. 13) also are predicted to have the greatest disruptive effects on the presumed helical structure of cytosolic loop3 and TM9 based on secondary structure predictions (results not shown).

**CFTR Activation by Nonhydrolyzable Nucleotides**

Any mutation that increases unliganded activity is predicted by a classical allosteric activation scheme to have “pleiotropic” effects on channel gating including enhanced activation by weak or partial agonists (16, 18, 22, 32). In this regard, GOF mutations of ligand-gated channels enhance activation by partial agonists by at least two related mechanisms. First, mutations that increase unliganded activity correspondingly increase the efficiencies of partial agonists to open the channel when they are bound to the channel, i.e. convert partial agonists into stronger agonists. Second, such mutations increase ligand sensitivity (occupancy) by biasing the equilibrium toward the higher affinity open state. Thus, GOF mutations also enhance ligand binding at normally sub-saturating concentrations. The slower deactivation (and presumably slower unbinding) after ATPγS removal that we observed for the K978C GOF mutant relative to wild type CFTR (Fig. 4) is consistent with the latter mechanism. We do not have direct evidence for enhanced binding of ATPγS or AMP-PNP by the GOF mutants in part because the poor activation of wild type CFTR precluded quantitative ATPγS and AMP-PNP titration experiments. But it seems likely that the GOF mutations increased CFTR activation by these analogs both by enhancing their binding at concentrations that normally may be sub-saturating for wild type CFTR as well as by increasing their efficiencies when they are bound to the channel.

ATPγS and AMP-PNP Interact Differently with the CFTR Channel—Although both ATPγS and AMP-PNP appear to behave as partial CFTR agonists, these nucleotides interact in different ways with the channel. Activation by either required both NBDs and was reduced (AMP-PNP) or eliminated (ATPγS) by the G551D signature sequence mutation in NBD1. However, AMP-PNP activation was far more inhibited by the corresponding signature sequence mutation in NBD2. In addition, ATPγS was effective at much lower concentrations, and unlike the case for AMP-PNP, the ATPγS titration curve for K978C-CFTR activation could be fit only by a two-binding site model with different affinities. These differences are perhaps not surprising. ATPγS typically binds with higher affinity to other ATPases and ABC transporters (34, 35). In addition, ATPγS binds asymmetrically to the two ATP binding sites in P-glycoprotein (ABCB1) with high and low affinities (K$_d$ values of 6 μM and 0.7 mM; Ref. 36) similar to that observed here for CFTR activation.

This apparent asymmetry in the two binding sites is different from the equivalent binding sites for a typical ligand-gated channel like the nAChR. In the case of AMP-PNP, we cannot rule out the possibility that the asymmetry is so great that AMP-PNP binds to (or is functionally effective at) only one of the two ATP binding pockets. There is biochemical and functional evidence that AMP-PNP can bind to both sites in the CFTR channel (4, 37, 38). It is well known that open channel bursts are prolonged by adding AMP-PNP in combination with ATP (15), which is most simply explained by the nonhydrolyzable analog replacing ATP at site 2 where it binds for many seconds in concert with stable ATP binding to site 1. When AMP-PNP is the sole ligand it may be functionally effective at only one of the sites, possibly site 2 given the argument above. But we cannot rule out the alternative possibility that in the absence of ATP...
this nucleotide analog is more effective at site 1 than 2. When both nucleotides are present, ATP binding to site 1 might allosterically increase the binding or efficacy of AMP-PNP at site 2. The notion that AMP-PNP might be functionally effective primarily at site 1 in the absence of ATP would be consistent with the stronger effect of the NBD2 signature sequence mutation (nearest site 1) on AMP-PNP activation of K978C-CFTR. This could also explain the normally weak activation by AMP-PNP alone given that ATP binding to site 2 appears to be functionally more significant in promoting wild type channel opening (3, 5, 6, 12). ATPγS in contrast appears to bind to and exert functional effects at both sites but with a lower apparent efficacy for channel opening than ATP.

GOF Mutations Rescue G551D and G1349D CF Channels but in Different Ways—The ABC signature sequences line the two ATP binding pockets and play important roles in ABC transporter function and CFTR channel gating. Their precise roles are unclear but probably relate to their participation in controlling NBD dimerization and/or signaling between the ATP binding pockets and the translocation pathway (39, 40). The G551D mutation in the NBD1 signature sequence abolishes ATP-dependent CFTR gating and causes severe CF. The analogous mutation in NBD2 (G1349D) causes less severe disease because it incompletely inhibits ATP-dependent gating (12). Previously we showed that the activity of the severely defective G551D mutant was increased substantially by GOF mutations such as K978C (13). But this elevated activity was due entirely to an increase in ATP-independent (unliganded) activity; we observed no rescue of ATP-dependent gating of the G551D mutant nor did we observe activation by ATPγS here. In contrast, we observed a more authentic rescue for the G1349D-K978C construct in the form of much greater ATP-dependent currents and lower relative activation by potentiators. This construct also exhibited larger ATP-independent currents, as expected for channels with the K978C GOF mutation. This more authentic rescue of the G1349D channel presumably relates to the incomplete loss of ATP-dependent gating caused by this signature sequence mutation versus the complete abolition of ATP dependence by the corresponding G551D mutation (12). G1349D-CFTR rescue by the K978C GOF mutation is analogous to its enhancement of CFTR activation by ATPγS or AMP-PNP. Each may be explained by a primary effect of the GOF mutation to increase unliganded activity with a consequent increase in ligand efficacy. In the case of AMP-PNP or ATPγS, the efficacy is compromised by the nature of the ligand itself. In the case of G1349D-CFTR, the signature sequence mutation in NBD2 may compromise the efficacy of ATP to open the channel.

Comparing CFTR Gating to the Gating of Conventional Ligand Channels—The present results provide additional support for the concept that CFTR channel gating obeys many of the allosteric principles that describe the gating of more typical ligand-gated channels. Aleksandrov and Riordan (41, 42) first argued that CFTR channel gating is allosterically coupled to ATP binding on the basis of CFTR activation by nonhydrolyzable analogs (albeit weak) and on thermodynamic arguments (although see Ref. 38). Loose coupling between CFTR gating and ATP hydrolysis also was proposed by Ramjeesingh et al. (43) on the basis of their mutational analysis of purified and reconstituted CFTR. These earlier proposals agree well with the more recent findings discussed above including the discovery of GOF mutations that increase ATP-free CFTR activity and promote activation by putative partial agonists. This view is further supported by (i) the discovery of natural compounds ((curcumin; Ref. 24) and drugs (VX-770; Ref. 44)) that promote ATP-independent CFTR channel activity and (ii) the observation that wild type channels open in the absence of ATP (10–13) as do channels that lack NBD2 and thereby cannot dimerize the NBDs (13, 23, 24). These multiple findings are inconsistent with a strict induced fit mechanism in which channel opening is obligatorily coupled to ATP binding (or NBD dimerization). Instead, they support an allosteric activation mechanism in which the open state is accessible in the absence of ligand and for which mutations and drugs can enhance channel activity by promoting ligand-free closed-to-open isomerizations.

Despite these similarities, there are obvious and important differences between the gating of CFTR channels and conventional ligand-gated channels like the nAChR. Quantitative differences include much slower closing rates for unliganded CFTR channels that result in spontaneous openings that last 2–3 orders of magnitude longer than for the nAChR (Refs. 13 and 16 and Fig. 2). This huge difference in gating kinetics presumably reflects more substantial conformational changes that underlie CFTR gating, probably due to its ABC transporter history. Qualitative differences include the aforementioned asymmetry in the two ligand binding sites for CFTR and the use of an enzymatic activity to speed ligand dissociation. Thus, CFTR appears to be a hybrid channel that blends the allosterism of a ligand-gated channel with the unique properties of binding site asymmetry and ATPase activity inherited from its ABC transporter ancestors.

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REFERENCES

1. Hwang, T. C., and Sheppard, D. N. (2009) Gating of the CFTR Cl− channel by ATP-driven nucleotide-binding domain dimerisation. J. Physiol. 587, 2151–2161
2. Vergani P, Lockless SW, Nairn AC, and Gadsby DC (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. Nature 433, 876–880
3. Berger, A. L., Ikuma, M., and Welsh, M. J. (2005) Normal gating of CFTR requires ATP binding to both nucleotide-binding domains and hydrolysis at the second nucleotide-binding domain. Proc. Natl. Acad. Sci. U.S.A. 102, 455–460
4. Aleksandrov, L., Aleksandrov, A. A., Chang, X. B., and Riordan, J. R. (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
5. Basso, C., Vergani, P., Nairn, A. C., and Gadsby, D. C. (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
6. Csángy, L., Vergani, P., and Gadsby, D. C. (2010) Strict coupling between CFTR’s catalytic cycle and gating of its Cl− ion pore revealed by distributions of open channel burst durations. Proc. Natl. Acad. Sci. U.S.A. 107, 1241–1246
CFTR Activation by Nonhydrolyzable Nucleotides

7. Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welsh, M. J., and Smith, A. E. (1991) Phosphorylation of the R domain by CAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* **66**, 1027–1036

8. Mathews, C. J., Tabcharani, J. A., Chang, X. B., Jensen, T. I., Riordan, J. R., and Hanrahan, J. W. (1998) Dibasic protein kinase A sites regulate bursting rate and nucleotide sensitivity of the cystic fibrosis transmembrane conductance regulator chloride channel. *J. Biol. Chem.* **273**, 365–377

9. Gunderson, K. L., and Kopito, R. R. (1995) Conformational states of CFTR associated with channel gating. The role ATP binding and hydrolysis. *Cell* **82**, 231–239

10. Vargani, P., Nairn, A. C., and Gadsby, D. C. (2003) On the mechanism of MgATP-dependent gating of CFTR Cl− channels. *J. Gen. Physiol.* **121**, 17–36

11. Hennager, D. J., Ikuma, M., Hoshi, T., and Welsh, M. J. (2001) A conditional probability analysis of cystic fibrosis transmembrane conductance regulator gating indicates that ATP has multiple effects during the gating cycle. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3594–3599

12. Bompadre, S. G., Sohma, Y., Li, M., and Hwang, T.-C. (2007) G551D and N1303 of CFTR in induced-fit conformational change in response to ATP binding. *J. Biol. Chem.* **282**, 255–265

13. Wang, W., Wu, J., Bernard, K., Li, G., Wang, G., Bevensee, M. O., and Kirk, K. L. (2010) ATP-independent CFTR channel gating and allosteric modulation by phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 3888–3893

14. Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell* **67**, 775–784

15. Gunderson, K. L., and Kopito, R. R. (1994) Effects of pyrophosphate and nucleotide analogs suggest a role for ATP hydrolysis in cystic fibrosis transmembrane regulator channel gating. *J. Biol. Chem.* **269**, 19349–19353

16. Auerbach A (2012) Thinking in cycles. MWC is a good model for acetylcholine receptor channels. *J. Gen. Physiol.* **129**, 285–298

17. Galzi, J. L., Edelstein, S. J., and Changeux, J. (1996) The multiple phenotypic effects of mutated receptors. *Trends Pharmacol. Sci.* **17**, 125–141

18. Chang, Y., and Weiss, D. S. (1999) Allosteric activation mechanism of the cyclic nucleotide-gated channel. *Annu. Rev. Physiol.* **61**, 775–801

19. Colquhoun, D. (1998) Binding, gating, affinity, and efficacy. The interpretation of structure–activity relationships for agonists and the effects of mutating receptors. *Br. J. Pharmacol.* **125**, 924–947

20. Grosman, C., and Auerbach, A. (2000) Kinetic, mechanistic, and structural aspects of unliganded gating of acetylcholine receptor channels. A single channel study of two transmembrane segment 12’ mutants. *J. Gen. Physiol.* **115**, 621–635

21. Cui, L., Aleksandrov, L., Chang, X. B., Hou, Y. X., He, L., Hagedus, T., Gentzsch, M., Aleksandrov, A., Balch, W. E., and Riordan, J. R. (2007) Domain interdependence in the biosynthetic assembly of CFTR. *J. Biol. Chem.* **282**, 981–994

22. Wang, W., Bernard, K., Li, G., and Kirk, K. L. (2007) Curcumin opens cystic fibrosis transmembrane conductance regulator channels by a novel mechanism that requires neither ATP binding nor dimerization of the nucleotide binding domains. *J. Biol. Chem.* **282**, 4533–4544

23. Szollosi, A., Vergani, P., and Csányi, L. (2010) Involvement of F1296 and N1303 of CFTR in induced-fit conformational change in response to ATP binding at NBD2. *J. Gen. Physiol.* **136**, 407–423

24. Bai, Y., Li, M., and Hwang, T. C. (2010) Dual roles of the sixth transmembrane segment of the CFTR chloride channel in gating and permeation. *J. Gen. Physiol.* **136**, 293–309

25. Wang, W., Oliva, C., Li, G., Holmgren, A., Lillig, C. H., and Kirk, K. L. (2005) Reversible silencing of CFTR chloride channels by glutathionylation. *J. Gen. Physiol.* **125**, 127–141

26. Zhou, M., Engel, A. G., and Auerbach, A. (1999) Serum choline activates mutant acetylcholine receptors that cause slow channel congenital myasthenic syndromes. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10466–10471

27. Moody, J. E., Millen, L., Birns, D., Hunt, J. F., and Thomas, P. J. (2002) Cooperative, ATP-dependent association of the nucleotide binding cassette domains during the catalytic cycle of ATP-binding cassette transporters. *J. Biol. Chem.* **277**, 21111–21114

28. Dulik, A. M., Bauer, C. B., Thoden, J. B., and Raymond, I. (1997) X-ray structures of the MgADP, MgATPγS, and MgAMPNP complexes of the Dicysteum discoidum myosin motor domain. *Biochemistry* **36**, 11619–11628

29. Sauna, Z. E., Kim, I. W., Nandigama, K., Kopp, S., Chiba, P., and Ambudkar, S. V. (2007) Catalytic cycle of ATP hydrolysis by P-glycoprotein. Evidence for formation of the ES reaction intermediate with ATP-γ-S, a nonhydrolyzable analogue of ATP. *Biochemistry* **46**, 13787–13799

30. Siányeva, A., Liu, R., and Sharam, F. J. (2010) Characterization of an asymmetric occluded state of P-glycoprotein with two bound nucleotides. Implications for catalysis. *J. Biol. Chem.* **285**, 7575–7586

31. Lewis, H. A., Buchanan, S. G., Burley, S. K., Connors, K., Dickey, M., Dorwart, M., Fowler, R., Gao, X., Guggino, W. B., Hendrickson, W. A., Hunt, J. F., Kearins, M. C., Lorimer, D., Maloney, P. C., Post, K. W., Rajashankar, K. R., Rutter, M. E., Sauder, J. M., Shrivver, S., Thibodeau, P. H., Thomas, P. J., Zhang, M., Zhao, X., Emtage, S. (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *EMBO J.* **23**, 282–293

32. Csányi, L., Nairn, A. C., and Gadsby, D. C. (2006) Thermodynamics of CFTR channel gating. A spreading conformational change initiates an irreversible gating cycle. *J. Gen. Physiol.* **128**, 523–533

33. Szentpétery, Z., Kern, A., Liliom, K., Sarkadi, B., Va´radi, A., and Bakos, E. (2004) The role of the conserved glycines of ATP-binding cassette signature motifs of MRPI in the communication between the substrate-biding site and the catalytic centers. *J. Biol. Chem.* **279**, 41670–41678

34. Payen, L., Gao, M., Westlake, C., Theis, A., Cole, S. P., and Deeley, R. G. (2005) Functional interactions between nucleotide binding domains and leukotriene C4 binding sites of multidrug resistance protein 1 (ABCC1). *Mol. Pharmacol.* **67**, 1944–1953

35. Aleksandrov, A. A., Aleksandrov, L. A., and Riordan, J. R. (2007) CFTR (ABCC7) is a hydrolysable-ligand-gated channel. *Pflugers Arch.* **453**, 692–702

36. Aleksandrov, A. A., and Riordan, J. R. (1998) Regulation of CFTR ion channel gating by MgATP. *FEBS Lett.* **431**, 97–101

37. Ramjesingh, M., Li, C., Garami, E., Huan L., Galley, K., Wang, Y., and Bear, C. E. (1999) Walker mutations reveal loose relationship between catalytic and channel-gating activities of purified CFTR (cystic fibrosis transmembrane conductance regulator). *Biochemistry* **38**, 1463–1468

38. Eckford, P. D., Li, C., Ramjesingh, M., and Bear, C. E. (2012) Cystic fibrosis transmembrane conductance regulator (CFTR) potentiator VX-770 (ivacaftor) opens the defective channel gate of mutant CFTR in a phosphorylation-dependent but ATP-independent manner. *J. Biol. Chem.* **287**, 36639–36649