Cysteine Residues in the Nucleotide Binding Domains Regulate the Conductance State of CFTR Channels

Melissa A. Harrington* and Ron R. Kopito†
*Department of Biology, Delaware State University, Dover, Delaware 19901; and †Department of Biological Sciences, Stanford University, Stanford, California 94305-5020 USA

ABSTRACT Gating of cystic fibrosis transmembrane conductance regulator (CFTR) channels requires intermolecular or interdomain interactions, but the exact nature and physiological significance of those interactions remains uncertain. Subconductance states of the channel may result from alterations in interactions among domains, and studying mutant channels enriched for a single conductance type may elucidate those interactions. Analysis of CFTR channels in inside-out patches revealed that mutation of cysteine residues in NBD1 and NBD2 affects the frequency of channel opening to the full-size versus a 3-pS subconductance. Mutating cysteines in NBD1 resulted in channels that open almost exclusively to the 3-pS subconductance, while mutations of cysteines in NBD2 decreased the frequency of subconductance openings. Wild-type channels open to both size conductances and make fast transitions between them within a single open burst. Full-size and subconductance openings of both mutant and wild-type channels are similarly activated by ATP and phosphorylation. However, the different size conductances open very differently in the presence of a nonhydrolyzable ATP analog, with subconductance openings significantly shortened by ATPγS, while full-size channels are locked open. In wild-type channels, reducing conditions increase the frequency and decrease the open time of subconductance channels, while oxidizing conditions decrease the frequency of subconductance openings. In contrast, in the cysteine mutants studied, altering redox potential has little effect on gating of the subconductance.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel with some unique biophysical properties. Because it belongs to a family of transporter proteins rather than ion channels (Ames and Lecar, 1992), the structure of CFTR is unlike that of other ion channels that have been studied. Moreover, the gating cycle of this channel is very complex, requiring hydrolysis of ATP at two different nucleotide binding domains (Anderson et al., 1991a; Bear et al., 1992; see Gadsby and Nairn, 1994, 1999 for reviews), as well as phosphorylation of the protein at many sites on a regulatory R domain (Tabcharani et al., 1991; Cheng et al., 1991; Rich et al., 1991). Under conditions resembling physiological, CFTR channels have a linear conductance of 7–10 pS (Egan et al., 1992; Tabcharani et al., 1993; Gunderson and Kopito, 1994). In addition, distinct subconductance states have been reported under various conditions in both patch clamp experiments and recordings from channels fused into planar lipid bilayers (Haws et al., 1992; McDonough et al., 1994; Xie et al., 1995; Gunderson and Kopito, 1995; Tao et al., 1996, Yue et al., 2000).

In one case an apparent subconductance was reported to be related to structural changes caused by the hydrolysis of ATP at the second nucleotide binding domain (Gunderson and Kopito, 1995). This subconductance has been shown to be related to a conformational shift in the channel that allows an open channel block by molecules of the buffer MOPS (Ishihara and Welsh, 1997). The open channel block that occurs with ATP hydrolysis causes a fast flickering in channel openings that results in an apparent change in the conductance (Gunderson and Kopito, 1995; Ishihara and Welsh, 1997).

Another type of subconductance was reported by Tao et al. (1996) who showed that wild-type CFTR channels display three distinct conductance states of approximately 8, 6, and 3 pS, respectively. According to Tao et al. (1996) channels showed both slow and fast conversions among the three conductance states, although openings to the 6-pS conductance state were relatively rare. Unlike the conductance transitions noted by Gunderson and Kopito (1995), transitions between the 8-pS conductance and the 3-pS conductance described by Tao et al. (1996) did not appear to depend on hydrolysis of ATP, as the transitions still occurred in the presence of nonhydrolyzable nucleotides.

The subconductance states observed by Tao et al. (1996) were present in both native and cloned CFTR channels, implying that the subconductance states were properties of the channel protein itself and not dependent on accessory proteins associated with channels in the native membrane. The presence of subconductance states in the CFTR channel has been proposed as evidence for intermolecular interactions such as a dimerization of the channel (Tao et al., 1996; Yue et al., 2000). In this model, the larger subconductance states of the channel are dependent on interdomain or intermolecular interactions, and disruption of those interactions leads to channels that open to the lower subconductances.

Submitted February 6, 2001, and accepted for publication December 7, 2001.

Address reprint requests to Dr. Melissa A. Harrington, Dept. of Biology, Delaware State University, 1200 North DuPont Highway, Dover, DE 19901. Tel.: 302-857-7117; Fax: 302-857-6512; E-mail: mharring@dsc.edu.

0006-3495/02/03/1278/15 $2.00

Although Tao et al. (1996) showed CFTR channels making transitions from one conductance state to the other, recently published results indicate that the small and large conductance are independent, and may result from separate, independently gating pores. Yue et al. (2000) report that CFTR molecules contain dual channel pores of separate conductance, one 9–11 pS and one ~4 pS. They base their conclusions on single channel data from CFTR truncation mutants. Channels that were truncated after the R domain opened only to the full-size conductance, while channels containing only the C-terminal half of the protein (with and without the R domain) opened only to the subconductance. Wild-type channels open to both conductances, each opening independently of the other (Yue et al., 2000). These results indicate that the pore structure and gating sequence of the CFTR channel involves a level of complexity not previously recognized. These properties of CFTR channels most likely depend on inter and intramolecular interactions that are just beginning to be elucidated.

Previous work with CFTR has shown that the oxidation state of the protein is an important regulator of channel gating kinetics, and that these effects are mimicked by reagents that modify the sulfhydryl moieties of cysteine residues (Stutts et al., 1994; Koettgen et al., 1996; Cotten and Welsh, 1997; Harrington et al., 1999). In reducing conditions, channel openings show frequent, short bursts of <1 s, while in oxidizing conditions channels open into much longer bursts that can last 5 min or more. Moreover, these long bursts are separated by long closed periods in which channels appear to be inactive (Harrington et al., 1999). Reducing conditions increase both the rate of channel opening (leading to more frequent openings) and the rate of channel closing (leading to shorter openings). Given the close linkage between ATP hydrolysis and channel gating, it seemed likely that redox-sensitive residues in the nucleotide binding domains would be involved in mediating this effect. Studies with P glycoprotein, an ABC-transporter protein closely related to CFTR, have shown that modification of cysteine residues in the nucleotide binding domains disrupts transporter function (Al Shawi et al., 1994; Loo and Clarke, 1995). Although redox modulation of cysteines does not disrupt channel activity in CFTR, it does alter the kinetics of channel gating, suggesting that the function of the nucleotide binding domains may be affected (Harrington et al., 1999). Because the CFTR nucleotide binding domains each contain two cysteines, it seemed logical that these residues might mediate the effects of redox potential on gating kinetics. Therefore, in this study we mutated four cysteine residues, two in NBD1 and two in NBD 2, to assess their role in redox-mediated changes in channel gating kinetics.

In this study we report that mutation of cysteine residues in NBD1 results in channels that open almost exclusively to a 3-pS subconductance, while the mutation of cysteines in NBD2 decrease the frequency of subconductance openings. Wild-type channels open to both size conductances and can make fast transitions between the two subconductance states within a single open burst. In addition, full-size and subconductance channels open very differently in the presence of ATPγS; subconductance openings are significantly shortened while full-size channels are locked open. The full-size and subconductance openings of both mutant and wild-type channels are similarly activated by ATP and phosphorylation; however, only wild-type subconductance channels show redox-mediated changes in gating kinetics. Mutation of the two cysteine residues in NBD2 decreases the frequency of subconductance openings and eliminates the effect of oxidation in increasing the burst durations of full-size channel openings.

METHODS

Patch clamp

Channels were recorded from inside-out patches pulled from HEK-293 cells transfected with wild-type and mutant human CFTR protein. Current traces were digitized at 500 Hz, with filtering at 100 Hz. Digitized data were analyzed with PCLAMP7 software (Axon Instruments, Foster City, CA) with filtering at 50 Hz. For burst duration analysis of full-size openings, the burst delimiter of 500 ms was determined from a plot of burst delimiter versus closings per burst as described in Sigurdson et al. (1987). Open dwell time and burst duration time constants were derived from fits of one or two exponentials using the maximum likelihood method. Burst and open time analysis was performed on patches with single channels or on unsuperimposed openings from multi-channel patches. During kinetic analysis, individual opening and closing events were accepted or rejected based on observation. This allowed the exclusion of noise and ensured that full-size and subconductance openings were analyzed separately even when they occurred in the same patch. To speed analysis, the shortest "flicker" events (those occurring in <10 ms) were excluded. The patch clamp buffer consisted of (in mM): 135 *N*-methyl-D-glucamine; ~135 HCl; 10 HEPES, 3 $MgCl_2$, pH 7.5. For inside-out patches, the solution bathing cytoplasmic face of the channel contained 1 mM ATP plus 5 mM $MgCl_2$, and channels were recorded with a pipette potential of 60–80 mV. Patches with low or no basal activity on excision were treated with 250 U/ml PKA before recording. Oxidizing conditions were generated by the addition of 100 μM phorbol 12-myristate 13-acetate (PMA), freshly prepared 100 μM $KMnO_4$, or 100 μM S-nitroso-*N*-acetyl-penicillamine (SNAP) dissolved in DMSO within 1 min of use. SNAP releases NO^+, which reacts both with sulfhydryl moieties to form oxidized nitrosothiols and with oxygen to form peroxynitrate, a strong oxidizer (Arnelle and Stamler, 1995). For kinetic analysis of channel events in oxidizing conditions, data were pooled from patches in which oxidizing conditions were established with different agents. This was done for two reasons: 1) the minutes-long open bursts of the wild-type channel in oxidizing conditions make it difficult to observe very many bursts within a single patch, so data were pooled from many different patches; 2) in oxidizing conditions subconductance openings are quite rare (one or two per minute), so data from many patches must be pooled. The oxidizing conditions were established using three different oxidizing agents, but effects of all agents used were fully reversible with β-ME, and it is probable that they had similar effects.

Site-directed mutagenesis and protein expression

Mutagenesis was performed using a PCR megaprimer mutagenesis strategy as described in Sarkar and Sommer (1990) and Ho et al. (1989). PCR primers were designed using the Primer program (Whitehead Institute for Medical Research, Cambridge, MA). All mutant sequences were confirmed

by DNA sequence analysis. Wild-type CFTR channels and those containing the C-QUAD-S mutation were stably transfected into HEK 293 cells. Briefly, the CFTR sequences were inserted into a pcDNA3.1 expression vector containing a neomycin resistance gene (Invitrogen, Carlsbad, CA). The vector was linearized with Nru I and transfected into HEK 293 cells using calcium phosphate (Graham and van der Eb, 1973). Cells were grown under G418 selection for four weeks. Clonal colonies were isolated, expanded, and tested for CFTR expression by Western blot with a C-terminal polyclonal CFTR antibody. Western blots of mutant CFTR channels showed large bands at ~160 kD, corresponding to the mature "band C" form (Cheng et al., 1990; Ward and Kopito, 1994). C491S, C524S, C1344/1355S, and C491/524S mutants were inserted into a pcDNA3.1 expression vector and transiently transfected into HEK 293 cells using calcium phosphate. Transfection efficiency was ~60%. Transfected cells were identified by the appearance of channel activity in cell-attached patches after the addition of forskolin to the bath. Wild-type and transfected HEK 293 cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine, and 500 μg/ml Geneticin (G418; for stably transfected cells). All tissue culture reagents were purchased from Gibco-BRL (Gaithersburg, MD).

RESULTS

Subconductance openings are frequent in wild-type CFTR channels

Examination of recordings from wild-type channels in inside-out patches revealed that openings to a 3-pS subconductance were common, and the subconductance appears to be the same as a 3-pS subconductance reported by Tao et al. (1996). Like the full-size channels, the subconductance channels require phosphorylation for high-probability opening (Fig. 1 *A*). In wild-type channels, subconductance openings have a conductance close to 3 pS, while the full-size openings have a conductance close to 8 pS (Fig. 1 *B*). In addition to the differences in conductance, subconductance channels have a shortened open dwell time as compared to full-size channels. As shown in Fig. 1 *C*, open dwell time histograms for both full-size and subconductance openings fall into two distributions. However, the time constants for the open dwell time distribution of the subconductance are about one-third of the full-size openings. The striking difference in gating kinetics for the full-size and subconductance openings indicates that the two types of openings result from different gating cycles, and perhaps even separate channel pores, as suggested by Yue et al. (2000).

Mutation of cysteine residues in NBD1 stabilize opening to a subconductance state

Previous work has shown that CFTR channel gating kinetics are modulated by redox potential. Because the kinetics of channel gating appear to be tightly regulated by the rate of ATP hydrolysis at the nucleotide binding domains (Li et al., 1996; Zeltwanger et al., 1999; Ikuma and Welsh, 2000), the modification of cysteine residues in these regions may affect channel gating. The CFTR protein has two cysteine residues in each nucleotide binding domain (NBD): C491 and C524 in NBD1, and C1344 and C1355 in NBD2. These cysteines were mutated to serines either singly or in groups. Because redox modulation alters channel gating kinetics, it was expected that mutating cysteine residues in regions intimately linked with channel gating would eliminate the effects of redox modulation. However, the effects of these mutations proved to be quite complex and unpredictable.

Mutating cysteine residues in the first nucleotide binding domain resulted in a striking change in channel gating behavior compared to wild-type channels. In inside-out patch clamp recordings, C491S mutant channels show openings to two different conductance levels, with the majority of channel openings to a subconductance of ~3 pS (Fig. 2, Table 1). Some patches also show openings to the full-size conductance (~8 pS), although these patches include openings to the smaller conductance as well. The subconductance openings of the C491S mutant are much shorter than the wild-type subconductance, as shown by comparing the dwell time histograms in Fig. 1 *C* with that in Fig. 2 *C*. Time constants for the two components of the open time distributions are about one-half those of the wild-type subconductance. Despite this difference, the C491S subconductance openings appeared to be very similar to the wild-type subconductance in current amplitudes and in requirement for phosphorylation. As shown in Fig. 3, C491S mutant channels, like the wild-type subconductance, require phosphorylation by PKA for high-probability openings.

A second mutant was constructed in which both cysteines in NBD1 were mutated to serine (C491/524S). While the C491S single mutation had occasional full-size openings along with subconductance openings, mutating both cysteine residues in NBD1 resulted in a channel that almost never opened to the full-size conductance (Table 1; Fig. 4, *A* and *B*). In inside-out patch recordings from a C491/524S double mutant, frequent subconductance openings were observed in 10 of 13 patches, while frequent full-size openings were observed in only one patch (Table 1).

A fourth, "C-QUAD-S" mutant was made in which all four cysteine residues in the nucleotide binding folds (C491, C524, C1344, and C1355) were mutated to serines. As shown in Fig. 4, *A* and *B* and Table 1, this mutant channel also opens almost exclusively to the subconductance state, with only occasional full-size openings observed in over 140 min of recording from 30 patches. In fact, the C-QUAD-S CFTR mutant is not easily activated at all. Even after PKA treatment, 11 of 30 patches from cells stably transfected with the mutant channel did not show "frequent openings" (five in 30 s) of either the full-size or the subconductance channels (Table 1). As shown by the dwell time histograms in Fig. 4 *C*, the long component of the open time distributions for the C491/524S and C-QUAD-S mutants is much shorter than the wild-type subconductance, and closer to that of the C491S mutant.

Mutation of C524 has little effect on channel gating

While CFTR channels carrying the C491S mutation either alone or in combination with C524S or C1344/1355S open almost exclusively to a 3 pS subconductance, channels carry-

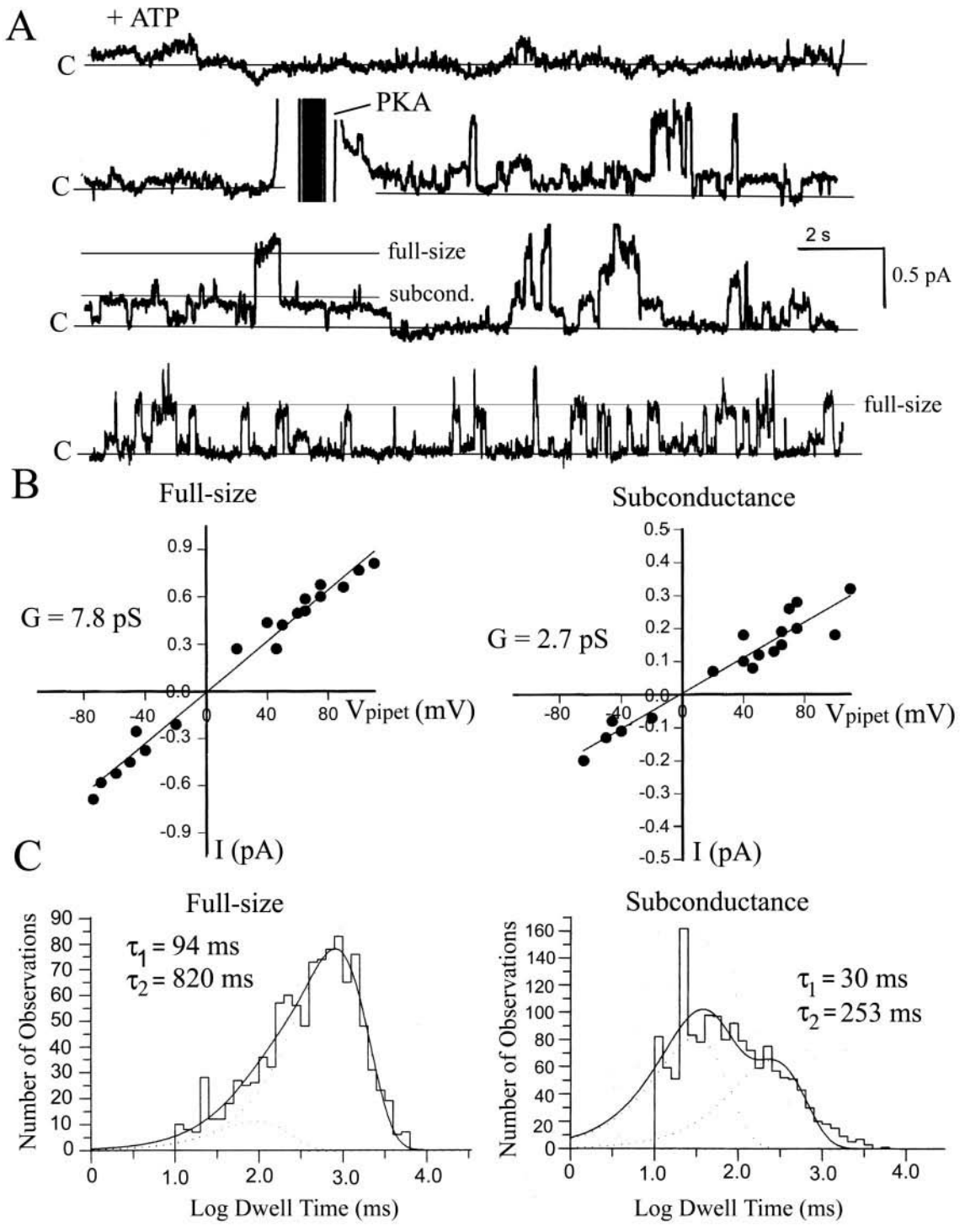

FIGURE 1 Wild-type CFTR channels gate to both 8-pS and 3-pS subconductances with different gating kinetics. (*A*) One-minute sample traces of inside-out patches expressing wild-type CFTR channels with and without PKA. After the addition of PKA, both full-size and subconductance channel openings are observed. Patch was held at 65 mV. (*B*) Current-voltage relationships of both full-size and subconductance openings of wild-type channels. Data are from three patches that displayed openings of both sizes. (*C*) Dwell time histograms of full-size and subconductance channels. Full-size channels, 2500 events; subconductance, 4000 events. Each dwell time distribution is fit with two exponential functions and the time constants (τ), are shown for each distribution.

ing only the C524S mutation exhibit conductance similar to wild-type channels. Like the wild-type channel, nearly every patch of C524S channels gates to the full-size openings, although, like wild-type channels, subconductance openings do appear (Table 1). Moreover, gating of the C524S mutant is sensitive to redox potential in a manner almost identical to that reported in wild-type channels, with the channel openings shortened by reducing conditions and oxidizing conditions resulting in long "locked open" bursts of the channel (Harrington et al., 1999).

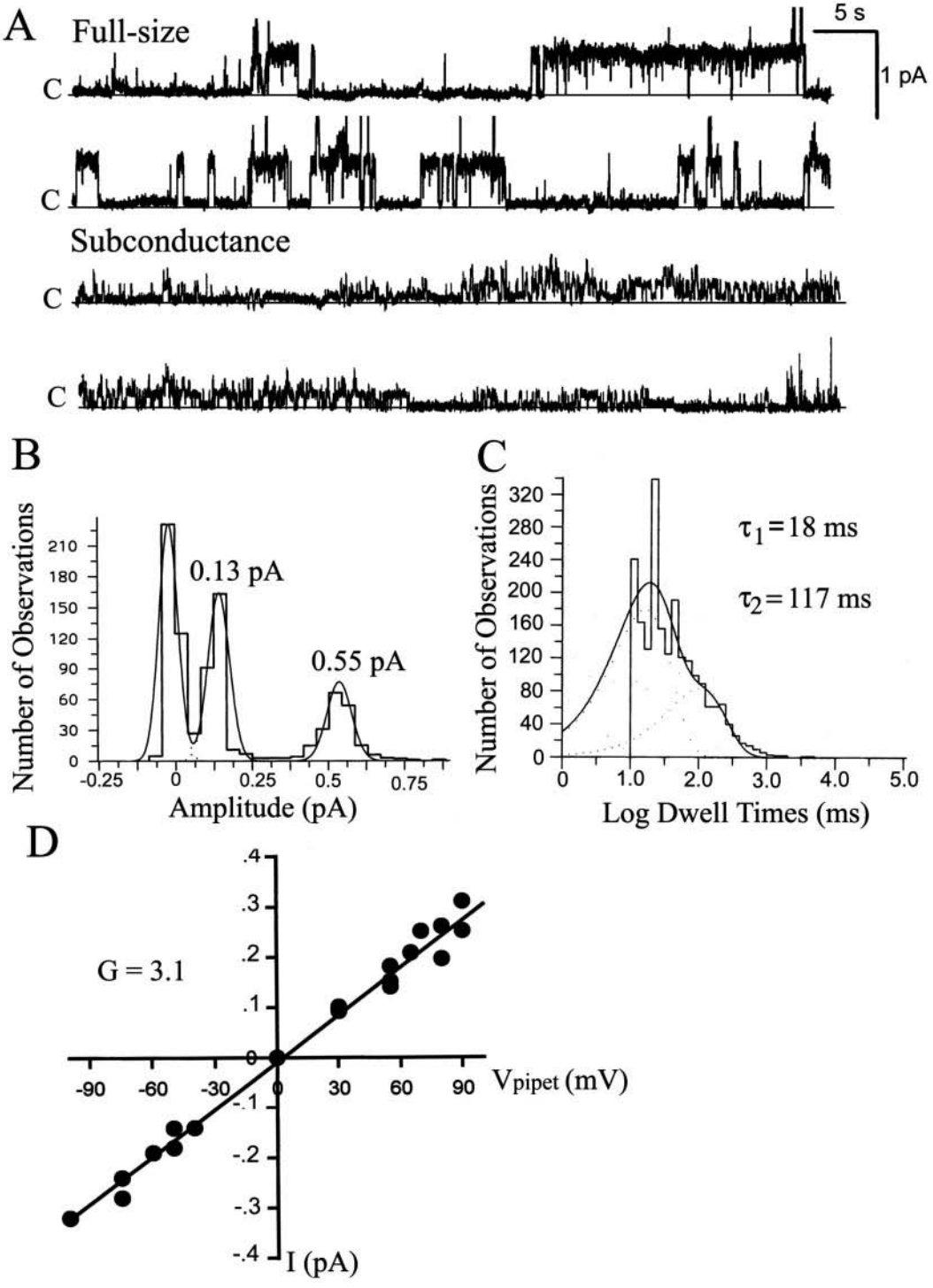

FIGURE 2 C491S-CFTR channels open most frequently to a 3-pS subconductance. (*A*) One-minute sample traces of inside-out patches from HEK 293 cells expressing the C491S mutant of CFTR showing the two modes of gating of the channel. The two lower traces are from a separate patch from the two upper traces. The patch was held at 70 mV for traces one and two, and 60 mV for traces three and four. (*B*) Amplitude histogram of 800 channel openings from the two patches shown in *A*. (*C*) Open dwell time histogram of 6000 opening and closing events of C491S channels in nine separate patches. The distribution is fit with two exponential functions and the time constants are shown for each distribution. (*D*) Current-voltage relationship of subconductance channels from C491S CFTR. Plot includes data from three separate patches.

Effect of nonhydrolyzable nucleotide analogs on subconductance openings

A striking difference in the behavior of the subconductance versus full-size openings in the wild-type channel is the effect of nonhydrolyzable ATP analogs on gating kinetics. In the presence of a mixture of ATP and ATPγS, full-size channels are "locked open": opening in long bursts that can last for minutes (Anderson et al., 1991b; Baukrowitz et al.,

TABLE 1 Mutations of cysteine residues in NBD1 increase the proportion of patches with subconductance openings, while mutations of cysteines in NBD2 decrease the proportion of patches with subconductance openings

Mutation	Patches with Subconductance	Patches with Full Size	Total Patches	Percent with Subconductance	Percent with Full Size
Wild-type	34	49	49	69	100
C491S	16	5	18	89	28
C491/524S	10	1	13	77	8
C524S	2	5	6	33	83
C1344/1355S	8	30	30	27	100
C-QUAD-S	19	0	30	63	0

The number of inside-out patches containing frequent full-size and/or subconductance channel openings were counted. "Frequent" channel openings were defined as being more than five openings in 30 s of continuous recording. Recordings from many patches included openings to both the full-size and subconductance state. For the C-QUAD-S mutant, many patches did not have frequent openings of either channel conductance.

1994; Hwang et al., 1994; Gunderson and Kopito, 1994). In contrast, in patch clamp experiments with channels exposed to a mixture of ATP and ATPγS (1 mM each), wild-type subconductance openings are dramatically shortened compared to openings in the presence of ATP alone (see Fig. 5). The long component of the dwell-time distribution is shortened by greater than a factor of two (Fig. 5 *B*). Moreover, the percentage of openings that fall into the longer distribution was reduced from one-third of the openings in ATP alone to less than one-quarter of the openings in a mixture of ATP and ATPγS. The difference between the distribution in the presence and absence of ATPγS was found to be significant for wild-type channels ($p < 0.05$; Kolmogorov-Smirnov). Two other mutants (C491S, C491/524S) were tested for the effect of ATPγS on subconductance openings (Fig. 5 *C*). Both the C491S and C491/524S mutants have open dwell times that are shorter than wild-type subconductance, and these dwell times were not significantly altered by the presence of ATPγS ($p > 0.05$; Kolmogorov-Smirnov).

These data show that when ATP hydrolysis at one or both NBDs is inhibited, the open state of the wild-type subconductance channel is much less stable, resulting in faster channel closing and shorter open bursts. This is in contrast

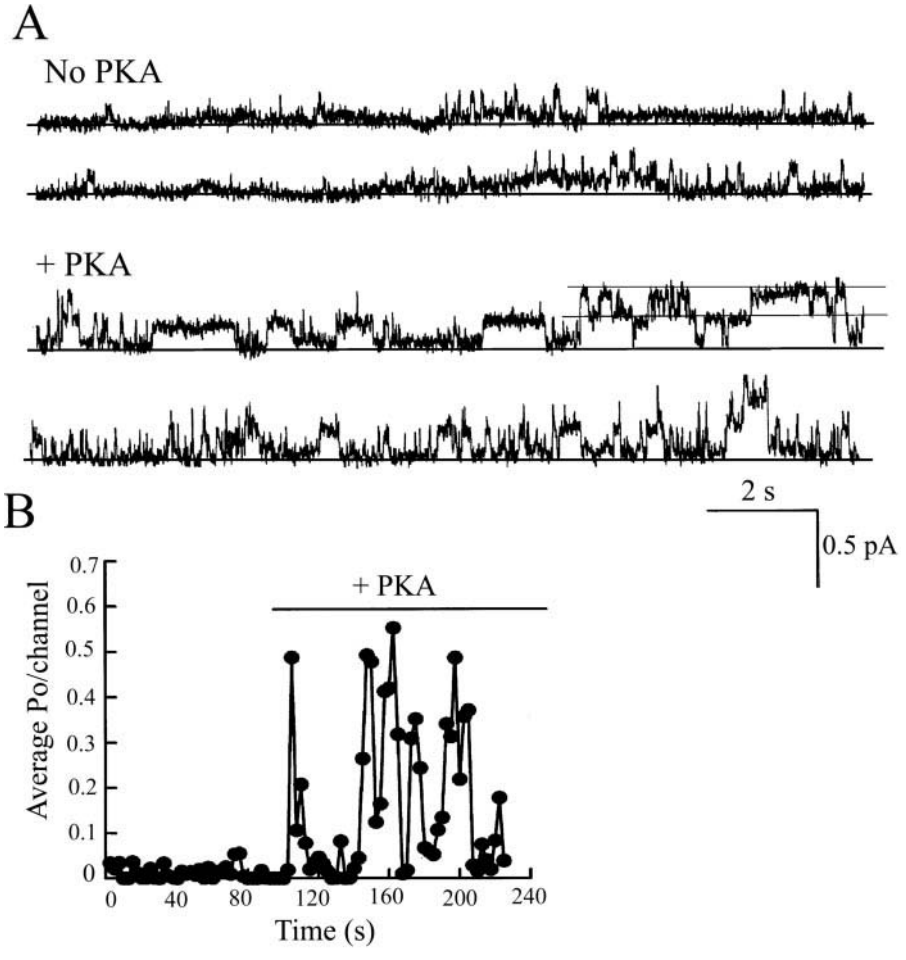

FIGURE 3 Increased phosphorylation by PKA increases the frequency of subconductance openings in C491S patches. (*A*) One-minute sample traces of an inside-out patch from an HEK 293 cell expressing C491S mutant CFTR with and without the addition of PKA. Note the increased length and frequency of openings after phosphorylation. Horizontal lines on trace 3 indicate openings of a second channel superimposed upon the first. The patch was held at 75 mV. (*B*) Graph of open probability versus time for the patch shown in *A*.

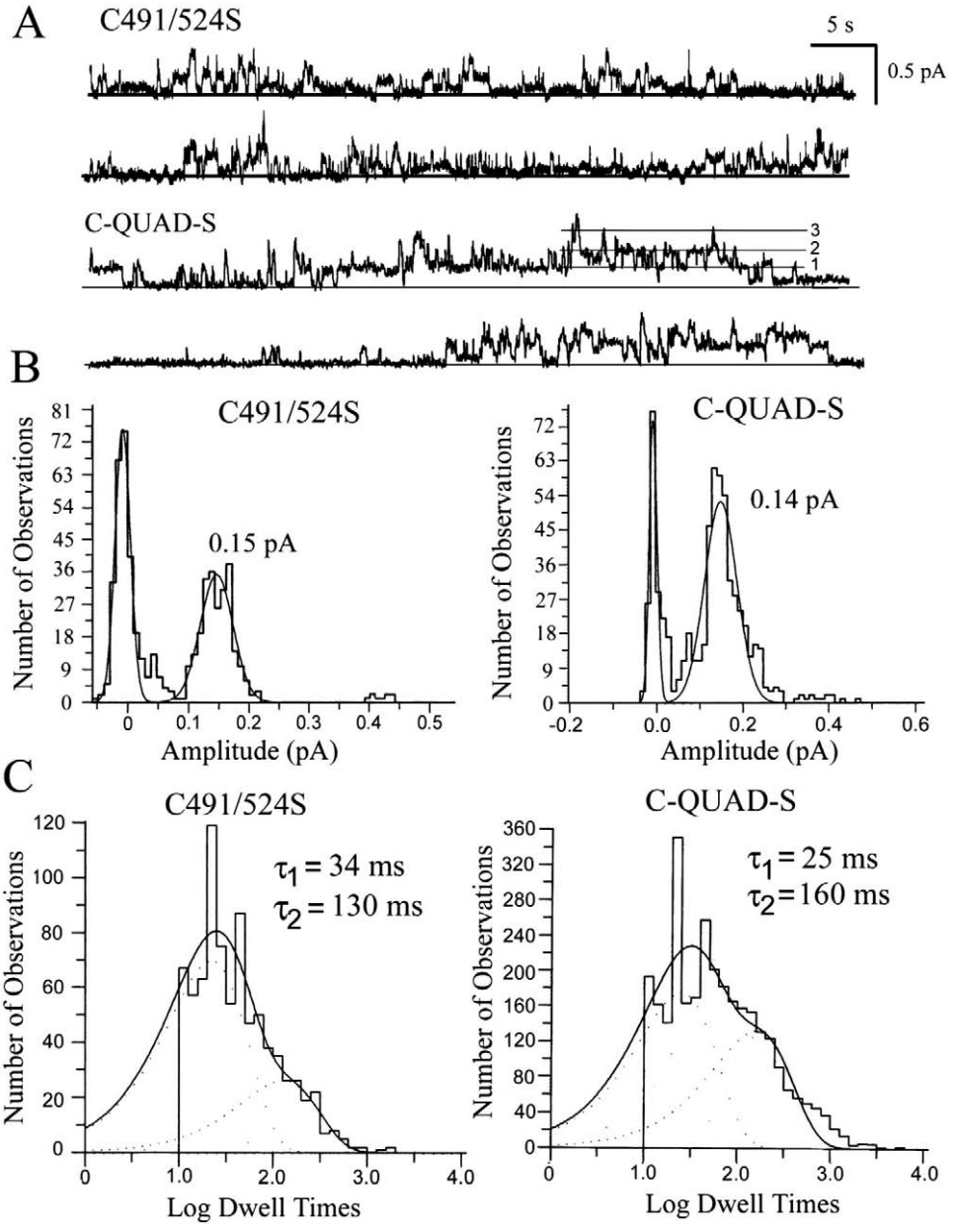

FIGURE 4 C491/524S and C-QUAD-S CFTR mutants open almost exclusively to 3 pS subconductance. (*A*) One-minute sample traces of inside-out patches from cells expressing C491/524S and C-QUAD-S mutant CFTR channels. Horizontal lines on the third trace indicate the superimposed openings of at least three channels. The patch was held at 65 mV. (*B*) Amplitude histograms of 500 channel openings from two patches containing the C491/524S mutant and two patches containing the C-QUAD-S mutant. (*C*) Open dwell time histograms of 3000 opening and closing events of the C491/524S mutant and 6000 events of the C-QUAD-S mutant. Each distribution is fit with two exponential functions and the time constants are shown for each distribution.

to full-size openings, where inhibiting hydrolysis of ATP greatly stabilizes channel opening, resulting in longer open bursts. Moreover, the presence of ATPγS shortens the open dwell time of wild-type channels to approximately that of channels containing the C491S mutant, suggesting that the altered behavior of the C491S channel may be related to changes in ATP hydrolysis at NBF1.

Effect of redox potential on gating of subconductance channels

Because CFTR channel gating is highly sensitive to changes in redox state, it was important to test the effect of oxidizing versus reducing conditions on the subconductance openings of wild-type channels and those with mutated cysteine residues. Subconductance openings from wild-type channels retain a sensitivity to reducing agents that is similar to that of full-size channels. In reducing conditions, the changes in opening and closing rates of the channel are similar in direction even though full-size and subconductance openings have quite different gating kinetics. As with the full-size openings, in the presence of 10 mM β-ME subconductance openings are shorter than in control conditions. This is illustrated in Fig. 6 *A* with a sample channel record from a patch containing wild-type channels in nonreducing conditions. In this patch the subconductance makes up the majority of the openings. The effect of the reducing agent on subconductance gating is quantified in Fig. 6 *B* with dwell time histograms showing that, in reducing conditions, the time constants of both distributions of the dwell time histogram are decreased. The difference in the distributions in the reduced and control conditions was found to be significant by the Kolmogorov-Smirnov test ($p < 0.05$).

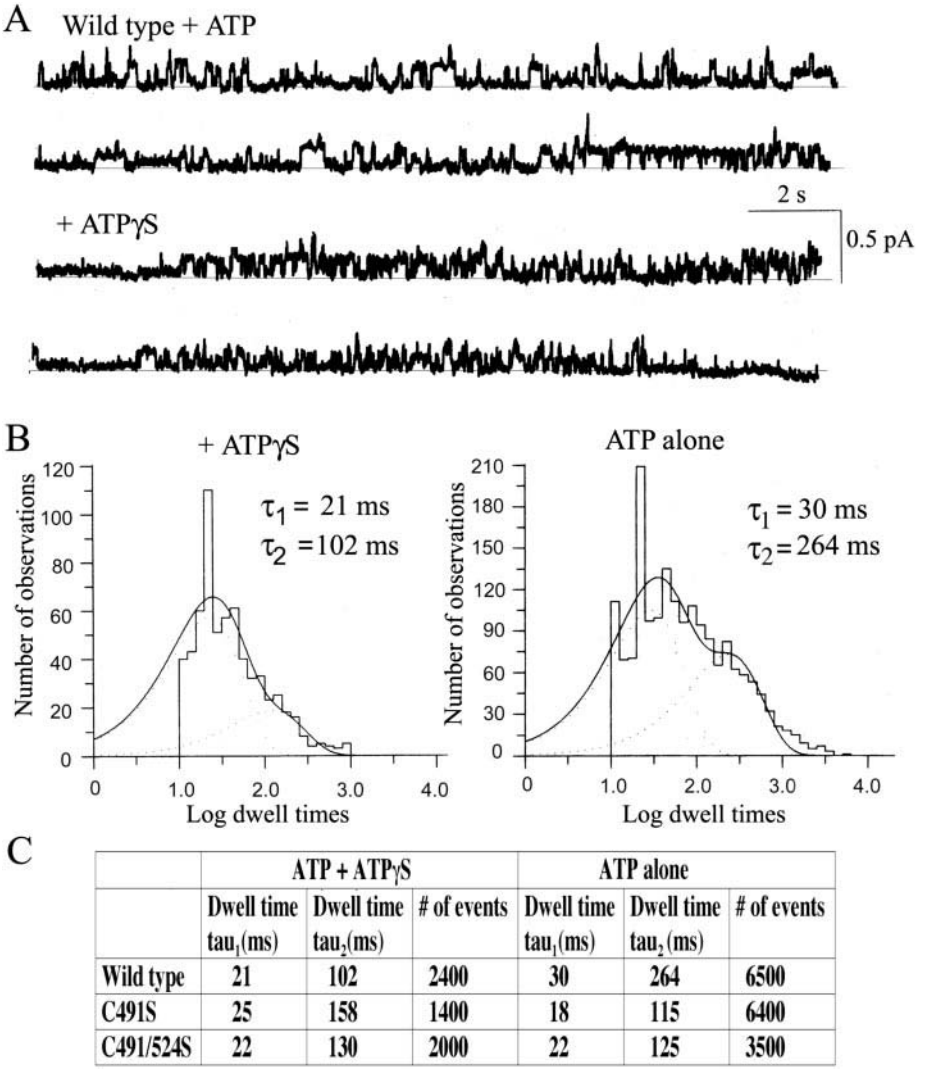

FIGURE 5 ATPγS shortens the open time of wild-type subconductance openings. (*A*) Thirty-second sample traces of inside-out patches showing wild-type CFTR channel openings in the presence and absence of ATPγS. The patch was held at 65 mV. (*B*) Dwell time histograms for 2500 wild-type subconductance events in the presence of ATPγS, and 5000 events in the presence of ATP alone. Each distribution is fit with two exponential functions and the τ values are shown for each distribution. The histogram distributions with and without ATPγS are significantly different ($p < 0.05$; Kolmogorov-Smirnov). (*C*) Table showing the time constants of wild-type and mutant CFTR channels in the presence and absence of ATPγS.

C

	ATP + ATPγS			ATP alone		
	Dwell time tau_1(ms)	Dwell time tau_2(ms)	# of events	Dwell time tau_1(ms)	Dwell time tau_2(ms)	# of events
Wild type	21	102	2400	30	264	6500
C491S	25	158	1400	18	115	6400
C491/524S	22	130	2000	22	125	3500

In contrast to its effect on full-size channels, oxidizing conditions had no effect on the gating kinetics of the subconductance of the wild-type channels as compared to nonreducing (Control) conditions (Fig. 6, *B* and *C*). These data suggest that for the subconductance, the faster gating kinetics require highly reduced conditions, such as in the presence of 10 mM β-ME. In less reducing conditions, such as the control and oxidized conditions, there is no difference in the gating of the subconductance channels. Also shown in Fig. 6 *C*, reducing agents had little effect on the gating kinetics of mutant subconductance channels, even for mutants in which reducing conditions altered the kinetics of full-size openings. Addition of 10 mM β-ME had the biggest effect on the C1344/1355S mutant, but the dwell time distribution from reducing conditions was not significantly different from the control ($p > 0.05$; Kolmogorov-Smirnov).

Changing redox potential alters the frequency of subconductance openings

In wild-type channels where the majority of patches display both full-size and subconductance openings, reducing conditions increase the likelihood of high-frequency subconductance openings, while oxidizing conditions decrease the frequency of subconductance openings. In patches containing wild-type channels, frequent subconductance openings (more than five in 30 s of recording) were observed in 30 of 31 patches treated with 10 mM β-ME, while frequent subconductance openings were seen in only 9 of 22 patches exposed to oxidizing conditions. Moreover, in 13 of 20 patches that were exposed first to oxidizing then reducing conditions, the frequent subconductance openings observed in the reducing conditions were absent after the patches were treated with oxidizing agents. A similar effect was observed with patches treated first with an oxidizing agent (PMA) followed by addition of 10 mM β-ME. Of the seven patches observed under first oxidized and then reduced conditions, only two showed frequent subconductance openings in oxidized conditions, while six of the seven showed frequent subconductance openings in the reducing conditions.

Fig. 7 shows 30-s sample traces from two different patches taken in the presence of 10 mM β-ME (reducing conditions) and the same patches in the presence of 100 μM SNAP, followed by the addition of β-ME to re-establish reducing conditions. In these sample traces subconductance openings that were frequent in the presence of β-ME were absent when

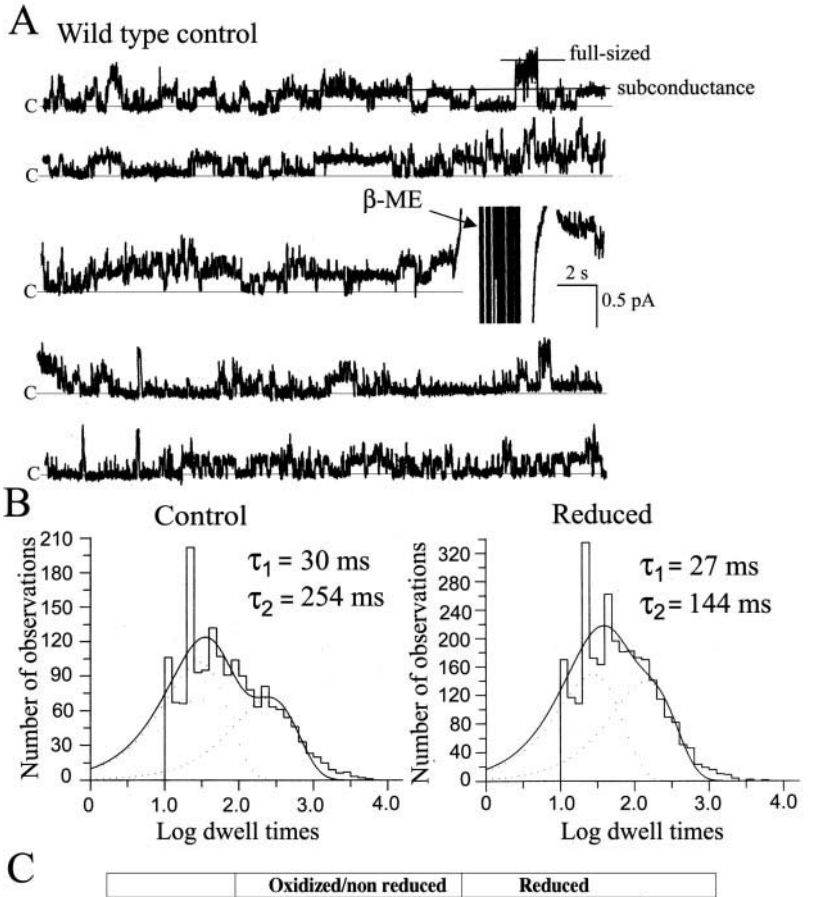

FIGURE 6 Reducing conditions shortens subconductance openings in wild-type, but not mutant, channels. (*A*) Thirty-second sample traces from a patch containing wild-type CFTR before and after the addition of 10 mM β-ME. Only a few full-sized openings are visible. The patch was held at 65 mV. (*B*) Open dwell time histograms of 6000 channel events in oxidized conditions (SNAP, PMA, and $KMnO_4$), and 7000 events in the presence of β-ME. Each distribution is fit with two exponential functions and the time constants are shown for each component. (*C*) Table showing the time constants of open dwell time distributions of subconductance openings from wild-type and mutant channels.

C

	Oxidized/non reduced			Reduced		
	Dwell time tau_1(ms)	Dwell time tau_2(ms)	# of events	Dwell time tau_1(ms)	Dwell time tau_2(ms)	# of events
Wild type (oxidized)	40	253	6500	27	144	7000
C491S	16	115	6500	26	138	4800
C524S	40	255	1500	26	254	1300
C1344/1355S	30	274	1800	54	204	2600
C-quad-S	24	144	4000	22	129	2200

the patch was treated with oxidizing agents, but reappeared when reducing conditions were restored. These data suggest that an oxidizing environment (and perhaps the formation of disulfide bonds) favors the type of interactions required for full-size openings, while reducing conditions allow greater subconductance openings.

Rapid transitions from full-size to subconductance in wild-type CFTR channels

Previous work has reported both slow conversions and rapid, spontaneous transitions between the full-size and two types of subconductance openings of wild-type CFTR channels (Tao et al., 1996). In our inside-out patch clamp recordings, CFTR channels make rapid transitions from the full-size to the subconductance and back, frequently within the same open burst (Fig. 8). These rapid transitions between full-size and subconductance were observed only in nonreducing conditions, where subconductance openings are rare and the open bursts of the full-size openings are prolonged. Fig. 8 includes sample traces from five different patches showing rapid transitions between the full-size and subconductance channels. In these traces filtered at 100 Hz, the subconductance openings appear as part of continuous open bursts of the full-size channel. In some cases the channel transitions to the subconductance without appearing to close, while sometimes it opens directly to the subconductance state and then transitions to the full-size channel. This type of rapid transition between full-size and subconductance was not observed in recordings of wild-type channels in reducing conditions, nor in the C491S, C491/524S, or C-QUAD-S mutants in which the subconductance state makes up the majority of channel openings observed. These transitions suggest that full-size and subconductance openings are not independent, but may rely on the same pore or pore structure.

Effect of mutation of C1344 and C1355 on subconductance frequency and redox sensitivity

Mutation of cysteine residues in the second nucleotide binding domain by themselves had much less of an effect on channel gating than the C491S mutation. Patches containing channels of the C1344/1355S mutant show predominantly a single con-

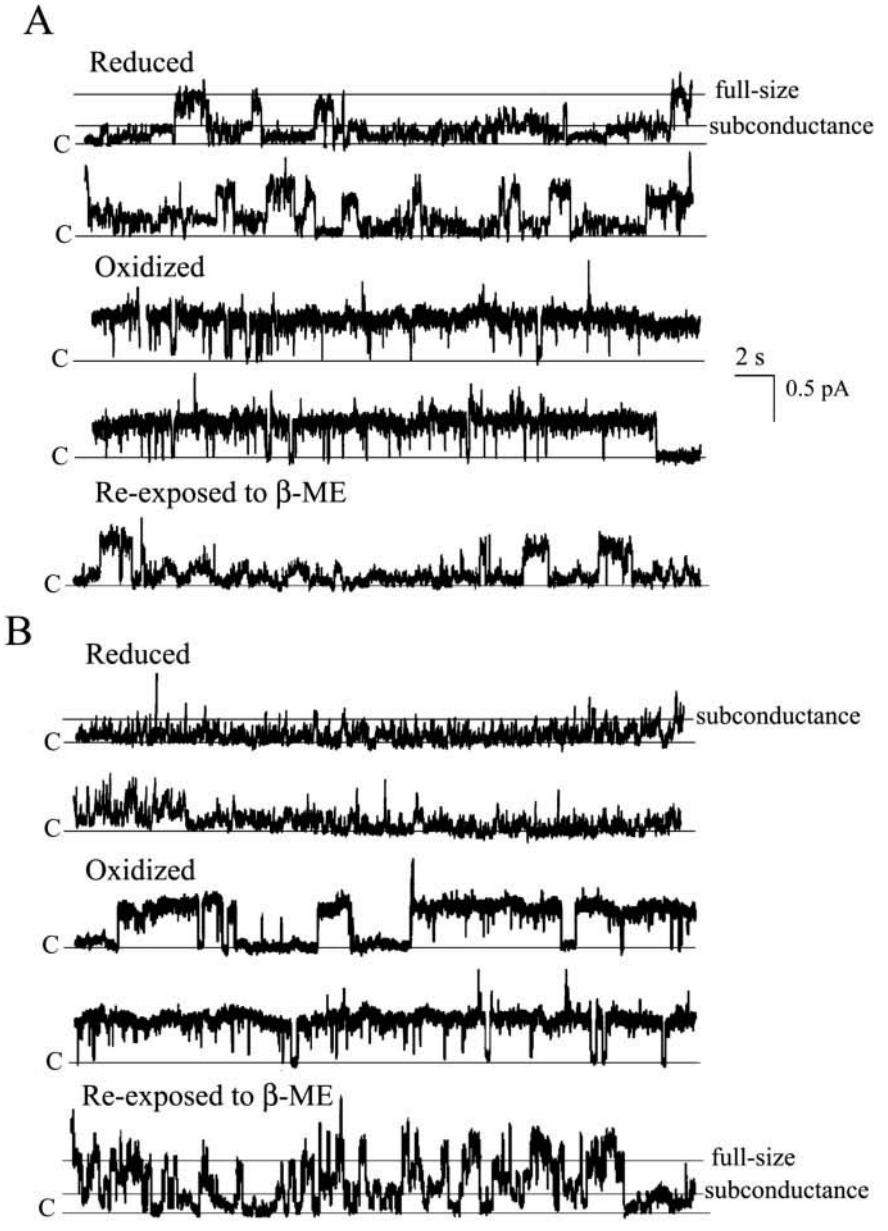

FIGURE 7 Reducing conditions increase the frequency of subconductance openings in wild-type channels. (*A*--*B*) Thirty-second sample traces from two different inside-out patches in both reduced and oxidizing conditions. Reducing conditions were established with 10 mM β-ME, while oxidizing conditions were in the presence of 100 μM SNAP. After treatment with SNAP, patches were returned to reducing conditions (10 mM β-ME). The patches were held at 65 mV.

ductance (8.3 pS; Fig. 9), and these channels require ATP and phosphorylation for gating just like the wild type (data not shown). However, unlike wild-type channels, subconductance openings were quite rare in this mutant, which gates almost exclusively to the full-size state (Table 1). In addition, the C1344/1355S mutant demonstrated subtle changes in the redox sensitivity of gating of the full-size channel. While reducing agents shortened channel openings and increased their frequency in a manner similar to the wild-type control, the C1344/1355S mutant channels were unaffected by oxidizing conditions that alter the burst properties of wild-type channels. As shown in Fig. 9, in the presence of reducing agents the burst duration histogram of both the wild-type and the C1344/1355S mutant falls into a single distribution with a time constant (τ) of $\sim$1 s. In control, nonreduced conditions, the burst duration histogram for both wild-type and mutant channels has two exponential components, with time constants of $\sim$1 s for the shorter burst component, and 6 s for the longer distribution (data not shown). In the presence of oxidizing agents, the longer component of the burst duration distribution of wild-type channels is shifted to the right, with a time constant of $>$19 s (Fig. 9, *C* and *D*). In contrast, the C1344/1355S mutant shows no increase in burst duration length in oxidized conditions as compared to control conditions. These results suggest that, while the cysteine residues in NBD2 do not mediate the effects of reducing agents on gating kinetics, they do play a role in allowing the strikingly long open bursts observed in oxidizing conditions.

DISCUSSION

This paper investigated the role of cysteine residues in the nucleotide binding domains of CFTR in modulating channel

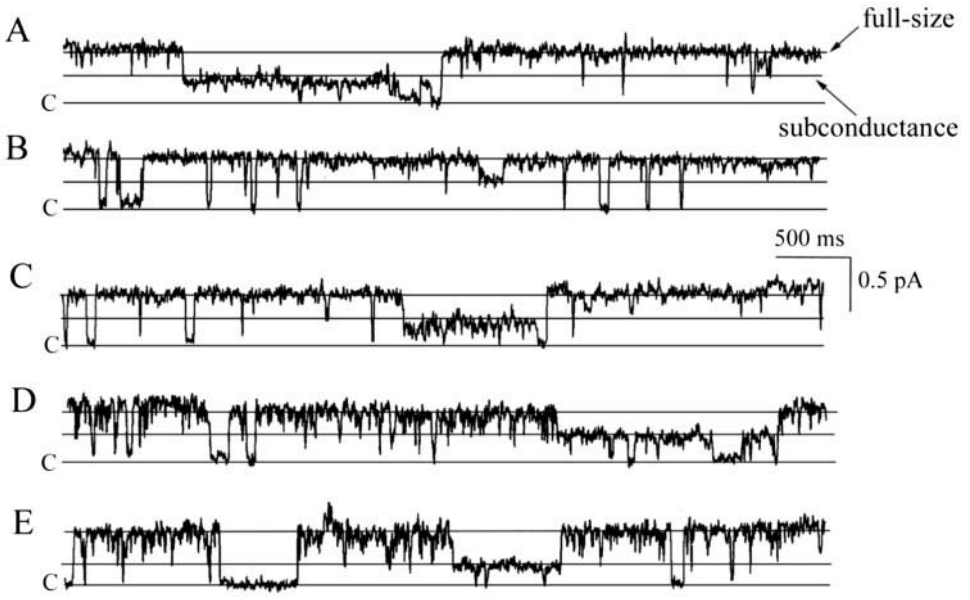

FIGURE 8 Wild-type channels make transitions between full-sized and subconductance openings within a single open burst. (*A*--*E*) Fifteen-second sample traces of wild-type channels from five different inside-out patches. Channels were pre-phosphorylated with PKA, which was then washed out. Channels were maintained in either oxidizing conditions with SNAP (*traces A*, *B*, *D*) or in nonreducing conditions in which redox potential was not controlled (*traces C* and *E*). Patches were held at 67–70 mV.

gating. Mutation of C491 in NBD1 to serine resulted in channels that opened almost exclusively to a 3-pS subconductance, while C524S mutant channels showed mostly full-size openings. In contrast, mutation of the two cysteine residues in the second nucleotide binding domain (C1344 and C1355) reduced the frequency of the subconductance openings compared to the wild-type channel.

The 3-pS subconductance of channels containing the C491S mutant alone or with other cysteine mutations was similar to the subconductance observed in recordings from patches containing wild-type CFTR channels, although with a shortened open dwell time. The 3-pS subconductance in both mutant and wild-type channels requires ATP and phosphorylation for high-probability opening just like the full-size channel, although the open times of subconductance openings are shortened compared to the full-size channel.

The effect of the C491S mutation on control of channel conductance (enriching for subconductance at the expense of full-size openings) was unexpected; however, the specificity of this mutation for that effect is supported by several pieces of data. The effect of redox modulation on the frequency of subconductance opening in the wild-type channel (shown in examples in Fig. 7, and described in Results), supports our hypothesis that C491 plays a critical role in regulating the conductance state of the channel. In wild-type CFTR, treatment with β-ME seem to promote subconductance opening, suggesting a role for cysteine residues in allowing subconductance openings to occur. In addition, the fact that mutation of another cysteine just 15 residues away (C524) has no effect on frequency of subconductance openings provides support for the unique role played by C491 in controlling the conductance state of CFTR.

Previous work has shown that the redox state of the channel has a large effect on the burst duration of CFTR channels, with reducing conditions resulting in a significant shortening of the burst durations of the full-size channels (Harrington et al., 1999). Even though the openings of the subconductance channel are short compared to full-size channels, in wild-type CFTR reducing conditions shorten the openings of subconductance openings just as they do for full-size channels. Interestingly, while strong reducing conditions shorten subconductance openings, the kinetics of the subconductance openings in oxidizing conditions are no different from the control (nonreduced) conditions. While the gating kinetics of full-size channels gets slower the more oxidized the protein, the kinetics of the subconductance change only in the most highly reduced conditions. These data support the hypothesis that subconductance and full-size openings result from different gating cycles that are differently affected by changes in redox potential.

Because the effects of changes in redox potential are presumably mediated through cysteine residues, mutating cysteine residues might be expected to alter the effect of redox potential on channel gating. In the case of the cysteines in the second nucleotide binding domain, substituting serine for cysteine did not alter the effect of reducing agents, but did eliminate the long open bursts in oxidizing conditions observed in the wild-type channel. These results indicate that multiple cysteine residues are involved in the effect of redox potential on channel gating kinetics. The cysteine residues in the second nucleotide binding domain appear to be important for the extraordinarily long bursts observed in oxidizing conditions, but other residues are involved in generating the very short bursts observed in strongly reducing conditions.

With channels containing the C491S mutation opening almost exclusively to a subconductance with gating properties very different from the full-size channel, it is difficult to directly relate the effect of redox potential on gating kinetics of the mutants compared to the wild-type channel. However, it appears that mutation of cysteine residues in the nucleotide binding domains eliminates redox-mediated

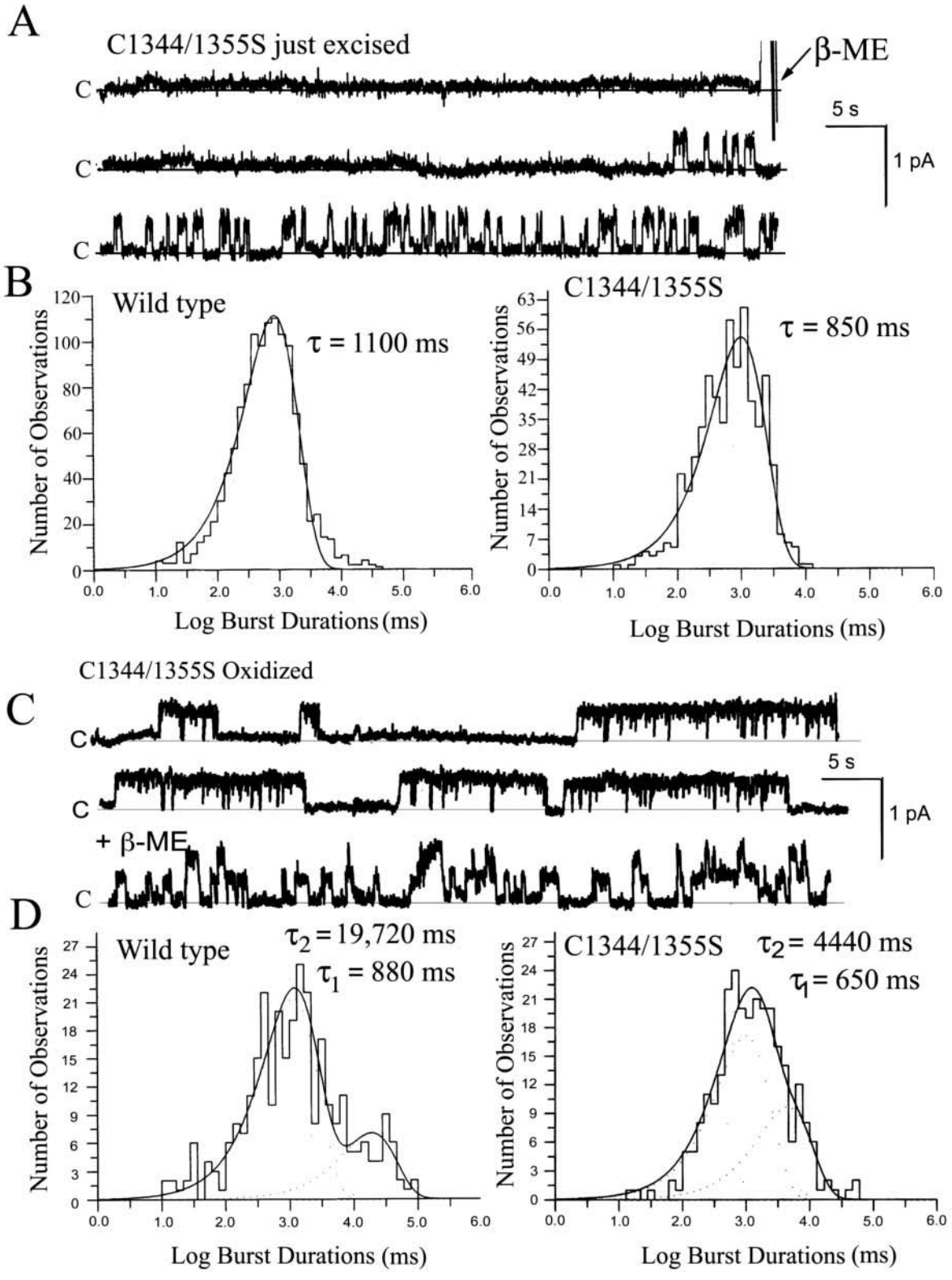

FIGURE 9 Burst duration of C1344/1355S mutant is shortened by reducing agents, but not changed by oxidizing agents. (*A*) Continuous 1-min sample traces from an inside-out patch from a C1344/1355S mutant CFTR channel before and after the addition of 10 mM β-ME. "C" indicates closed state of the channel. The patch was held at 65 mV. (*B*) Burst duration histograms of 6000 events each from wild-type and C1344/1355S mutant channels in the presence of 10 mM β-ME. For both channel types the distributions are fitted by a single exponential. In control conditions (redox potential unregulated), each distribution is fit with two exponential functions and the time constants (τ) are as follows: wild-type, τ_1 = 1180 ms, τ_2 = 7660 ms; for the C1344/1355S mutant τ_1 = 750 ms; τ_2 = 6060 ms. (*C*) Continuous 1-min sample traces from a patch under oxidizing conditions (100 μM SNAP), followed by administration of β-ME to restore reducing conditions. Patch was held at 65 mV. (*D*) Burst duration histograms of 5000 events each from wild-type and C1344/1355S mutant in oxidizing conditions (in the presence of SNAP, $KMnO_4$, or PMA). Each distribution is fit with two exponential functions and the time constants (τ) are shown for each distribution.

changes in the open time of subconductance channels. Wild-type subconductance openings are shortened by reducing agents, while none of the open time distributions from the cysteine mutants was significantly altered by changes in redox potential.

The subconductance openings of both the mutant and wild-type channels display activation properties that are very similar to the full-size channels—they require ATP and phosphorylation to open, although their gating kinetics are very different. Because gating of the full-size channel appears to be closely linked to ATP hydrolysis by the nucleotide binding domains (Li et al., 1996; Zeltwanger et al., 1999; Ikuma and Welsh, 2000), the large difference in kinetics indicates that subconductance openings are not related to ATP hydrolysis in quite the same way as the full-size openings. Further evidence for this difference is provided by the difference in the kinetics of subconductance versus full-size channels in the presence of the nonhydrolyzable nucleotide ATPγS. Full-size channels are "locked-open" by ATPγS, resulting in very long open bursts. In contrast, subconductance openings from wild-type channels are dramatically shortened in the presence of ATPγS. Because mutations of the channel that slow ATP hydrolysis by the second nucleotide binding domain also result in "locked-open" channels like those seen with ATPγS, it has been assumed that the long openings in the presence of nonhydrolyzable ATP analogs correspond to block of ATP hydrolysis by the second nucleotide binding domain. However, as ATPγS does not lock open the subconductance channels, but rather shortens their opening, the subconductance state appears to represent a significant alteration of the relationship between nucleotide hydrolysis and opening of the pore. One possibility is that subconductance openings represent a mode of gating in which ATP binding and hydrolysis do not occur in one of the nucleotide binding domains. Our data suggest that subconductance opening may be related to a decreased hydrolysis by the first nucleotide binding domain because mutation of C491S in NBD1 results in an increase in subconductance frequency at the expense of the full-size openings.

Recently published work with truncation mutants of CFTR has suggested that the CFTR channel might be "double-barreled," with one pore producing the full-size conductance and a second, independently gated pore producing a 3–4-pS subconductance (Yue et al., 2000). This hypothesis is based on data demonstrating that full-size openings are produced by channels consisting of only the amino portion of the protein (up to and including the R domain), while the subconductance appears in recordings of channels containing only the carboxyl portion of the protein from the R domain to the end (Yue et al., 2000). The current results provide support for the double-barreled model as mutation of the two cysteines in NBD1 nearly eliminates full-size openings, while mutation of the cysteines in NBD2 decreases the frequency of the subconductance openings. In addition, the finding of Yue et al. (2000), that subconductance openings occur in the absence of the first nucleotide binding domain, provides support for the hypothesis that subconductance openings in wild-type channels occur in the absence of hydrolysis at NBD1. However, in contrast to the Yue et al. (2000) report that the two pores in the channel gate independently, our results show rapid transitions from a full-size opening to a subconductance opening and back again within a single open burst. Because the channels transition to the subconductance without appearing to close (at least the closings are not detectable at 100-Hz filtering), the two types of openings probably do not result from different channel proteins, or even two independent pores of the same channel. Although the complete CFTR channel may contain two pores, the current results suggest that the pores are not completely independent.

Our data show that treating wild-type channels with reducing agents increases the frequency of subconductance openings observed, while oxidizing conditions decrease the frequency of subconductance openings. Recently published work with tandem linked dimers of CFTR demonstrated that dimers produce a single functional channel with full-size openings (Zerhusen et al., 1999). These results indicate that the full-size channel openings may require a dimer of the CFTR protein or a dimer of the amino half of the protein (see Yue et al., 2000). Additional support for the involvement of multiple CFTR proteins in channel gating was provided by Wang et al. (2000), who showed that linking of two or more channels via an accessory protein increased Cl^- channel activity. Wang et al. (2000) used two types of linking proteins, a bivalent monoclonal antibody and a hydrophilic CFTR binding protein named CAP70. CAP70 can bind as many as three CFTR molecules and promote the intermolecular contact that is associated with potentiation of channel activity. The frequency of subconductance openings in CFTR channels with the C491S mutation may mean that this region of the molecule is important for the type of intermolecular interactions observed by other groups to be important for high-frequency opening of the full-size channel. It is conceivable that the cysteine residues in NBD1, particularly C491, could be important in the intermolecular associations that stabilize a dimer of the channel and allow it to produce full-size openings. Channels in which C491 and C524 are mutated may be less able to adapt the conformation necessary for full-size openings, while the ability to generate subconductance openings is unaffected.

The mechanism by which C491 or C524 could stabilize such an intermolecular or interdomain interaction is not clear. These residues are almost certainly within the cytoplasmic domain of the channel, and it is questionable whether cysteines at this site could form disulfide bonds in physiological conditions. However, recent work examining gel mobility shifts of wild-type and mutant CFTR channels exposed to oxidizing conditions indicates that it is likely that one or more of these residues will form a disulfide bond in oxidizing conditions. Kembi and Harrington (2001) have shown that the mobility of wild-type channels is decreased in oxidized conditions, consistent with formation of a disulfide bond, while C491/524S and C-QUAD-S mutants show the same gel mobility in both oxidized and reduced conditions. Although the changes in gel mobility were only

observed in a strongly oxidizing environment, and may not be relevant to physiological conditions, earlier published experiments have shown that CFTR channels in cell-attached patches are not uniformly in a reduced state (Harrington et al., 1999). In addition, other research has shown that cysteine residues in proteins form disulfide bonds based on the redox potential of the bond, and it is possible to have a disulfide bond with a higher redox potential than is found in cell cytoplasm (Feng and Forgac, 1994), and the sulfhydryl groups of such cysteine residues could be oxidized even in cell cytoplasm. Taken together, these results make it conceivable that oxidation of cysteine residues plays a role in regulation of channel gating in vivo.

Other support for a possible role of disulfide bond formation in channel regulation comes from recent work with a voltage-gated K^+ channel that has indicated that regulation of the redox potential and membrane potential of cells may somehow be coupled. Gulbis et al. (1999) showed that the β-subunit of the *Shaker* voltage-dependent K^+ channels is an oxidoreductase with an NADPH (the reduced form of nicotinamide adenine dinucleotide) cofactor. Although the function of this oxidoreductase is not yet known, the four transmembrane channel subunits co-assemble into a permanent complex with four cytoplasmic β-subunits, indicating that the β-subunits are critical for the normal functioning of the channel (Gulbis et al., 2000). Although there is, as yet, no evidence that the CFTR channel associates with a similar protein, these results with the *Shaker* channel point to a previously unsuspected role for redox potential in regulating ion channel function. In the CFTR channel, redox-sensitive residues appear to affect both the kinetics of channel gating and transitions between subconductance states. Inside-out patch recordings from CFTR channels containing mutated cysteine residues provide additional evidence that the two nucleotide binding domains play very different roles in channel gating and permeation, and may even be part of separate channel pores.

This work was supported by a grant from the Cystic Fibrosis Foundation. M.A.H. was supported by a post-doctoral fellowship from the National Institute of Diabetes, Digestive and Kidney Diseases (DK09717) and Grants MCB-9874490 and HRD-9815529 from the National Science Foundation.

REFERENCES

Ames, G. F.-L., and H. Lecar. 1992. ATP dependent bacterial transporters and cystic fibrosis: analogy between channels and transporters. *FASEB J.* 6:2660–2666.

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

Anderson, M. P., R. J. Gregory, S. Thompson, D. W. Souza, S. Paul, R. C. Mulligan, A. E. Smith, and M. J. Welsh. 1991b. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science.* 253:202–204.

Arnelle, D. A., and J. S. Stamler. 1995. NO^+, NO, and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.* 318:279–285.

Al Shawi, M. S., I. L. Urbatsch, and A. E. Senior. 1994. Covalent inhibitors of P-glycoprotein ATPase activity *J. Biol. Chem.* 269:8986–8992.

Baukrowitz, T., T.-C. Hwang, A. C. Nairn, and D. C. Gadsby. 1994. Coupling of CFTR Cl^- channel gating to an ATP hydrolysis cycle. *Neuron.* 12:473–482.

Bear, C. E., C. H. Li, N. Kartner, R. J. Bridges, T. J. Jensen, M. Ramjeesingh, and J. R. Riordan. 1992. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell.* 68:809–818.

Cheng, S. H., R. J. Gregory, J. Marshall, S. Paul, D. W. Souza, G. A. White, C. R. O'Riordan, and A. E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell.* 63:827–834.

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

Cotten, J. F., and M. J. Welsh. 1997. Covalent modification of the regulatory domain irreversibly stimulates cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 272:25617–25622.

Egan, M., T. Flotte, S. Afione, R. Solow, P. L. Zeitlin, B. J. Carter, and W. B. Guggino. 1992. Defective regulation of outwardly rectifying Cl^- channels by protein kinase A corrected by insertion of CFTR. *Nature (Lond.).* 358:581–584.

Feng, Y., and M. Forgac. 1994. Inhibition of vaculolar H^+-ATPase by disulfide bond formation between cysteine 254 and cysteine 532 in subunit A. *J. Biol. Chem.* 269:13224–13230.

Gadsby, D. C., and A. C. Nairn. 1994. Regulation of CFTR channel gating. *Trends Biochem. Sci.* 19:513–518.

Gadsby, D. C., and A. C. Nairn. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev.* 79:S77–S107.

Graham, F. L., and A. J. van der Eb. 1973. Transformation of rat cells by DNA of human adenovirus 5. *Virology.* 54:536–539.

Gulbis, J. M., S. Mann, and R. MacKinnon. 1999. Structure of a voltage-dependent K^+ channel β subunit. *Cell.* 97:943–952.

Gulbis, J. M., M. Zhou, S. Mann, and R. MacKinnon. 2000. Structure of the cytoplasmic subunit–T1 assembly of voltage-dependent K^+ channels. *Science.* 289:123–127.

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

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

Harrington, M. A., K. G. Gunderson, and R. R. Kopito. 1999. Divalent cations and redox reagents alter gating of the CFTR channel. *J. Biol. Chem.* 274:27536–27544.

Haws, C. M., M. E. Krouse, Y. Xia, D. C. Gruenert, and J. J. Wine. 1992. CFTR Cl^- channels in immortalized human airway cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 263:L692–L707.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 77:51–59.

Hwang, T.-C., G. Nagel, A. C. Nairn, and D. C. Gadsby. 1994. Regulation of the gating of the cystic fibrosis transmembrane conductance regulator Cl channels by phosphorylation and ATP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 91:4698–4702.

Ikuma, M., and M. J. Welsh . 2000. Regulation of CFTR Cl^- channel gating by ATP binding and hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 97:8675–8680.

Ishihara, H., and M. J. Welsh. 1997. Block by MOPS reveals a conformational change in the CFTR pore produced by ATP hydrolysis. *Am. J. Physiol. Cell Physiol.* 273:C1278–C1289.

Kembi, F., and M. A. Harrington. 2001. Interdomain but not intermolecular interactions observed in CFTR channels. *Biochem. Biophys. Res. Commun.* 288:819–826.

Koettgen, M., A. E. Busch, M. J. Gregor, and K. Kunzelmann. 1996. N-Acetyl-L-cysteine and its derivatives activate a Cl^- conductance in epithelial cells. *Pfluegers Arch. Eur. J. Physiol.* 431:548–555.

Li, C., M. Ramjeesingh, W. Wang, E. Garami, M. Hewryk, D. Lee, J. M. Rommens, K. Galley, and C. E. Bear. 1996. ATPase activity of the cystic

fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 271: 28463–28468.

Loo, T. W., and D. M. Clarke. 1995. Membrane topology of a cysteine-less mutant of human P-glycoprotein. *J. Biol. Chem.* 270:843–848.

McDonough, S., N. Davidson, H. A. Lester, and N. A. McCarty. 1994. Novel pore-lining residues in CFTR that govern permeation and open channel block. *Neuron.* 13:623–634.

Rich, D. P., R. J. Gregory, M. P. Anderson, P. Manavalan, A. E. Smith, and M. J. Welsh. 1991. Effect of deleting the R domain on CFTR-generated chloride channels. *Science.* 253:205–207.

Sarkar, G., and S. S. Sommer. 1990. The "Megaprimer" method of site directed mutagenesis. *Biotechniques.* 8:404–407.

Sigurdson, W. J., C. E. Morris, B. L. Brezden, and D. R. Gardner. 1987. Stretch activation of a K^+ channel in molluscan heart cells. *J. Exp. Biol.* 127:191–209.

Stutts, M. J., S. E. Gabriel, E. M. Price, B. Sarkadi, J. C. Olsen, and R. C. Boucher. 1994. Pyridine nucleotide redox potential modulates cystic fibrosis transmembrane conductance regulator Cl^- conductance. *J. Biol. Chem.* 269:8667–8674.

Tabcharani, J. A., J. M. Rommens, Y.-X. Hou, X.-B. Chang, L.-C. Tsui, J. R. Riordan, and J. W. Hanrahan. 1991. Phosphorylation-regulated Cl channel in CHO cells stably expressing the cystic fibrosis gene. *Nature (Lond.).* 352:628–631.

Tao, T., J. Xie, M. L. Drumm, J. Zhao, P. B. Davis, and J. Ma. 1996. Slow conversions among subconductance states of cystic fibrosis transmembrane conductance regulator chloride channel. *Biophys. J.* 70:743–753.

Wang, S., H. Yue, R. B. Derin, W. B. Guggino, and M. Li. 2000. Accessory protein facilitated CFTR-CFTR interaction: a molecular mechanism to potentiate chloride channel activity. *Cell.* 103:169–179.

Ward, C. L., and R. R. Kopito. 1994. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 269: 25710–25718.

Xie, J., M. L. Drumm, J. Ma, and P. B. Davis. 1995. Intracellular loop between TM4 and TM5 of CFTR is involved in regulation of chloride channel conductance state. *J. Biol. Chem.* 279:28084–28091.

Yue, H., S. Devidas, and W. B. Guggino. 2000. The two halves of CFTR form a dual pore ion channel. *J. Biol. Chem.* 275:10030–10034.

Zeltwanger, S., F. Wang, G. T. Wang, K. D. Gillis, and T. C. Hwang. 1999. Gating of the cystic fibrosis transmembrane conductance regulator chloride channels by adenosine triphosphate hydrolysis: quantitative analysis of gating schemes. *J. Gen. Phys.* 113:541–554.

Zerhusen, B., J. Zhao, J. Xie, P. B. Davis, and J. Ma. 1999. A single conductance pore for chloride ions formed by two cystic fibrosis transmembrane conductance regulator molecules. *J. Biol. Chem.* 274: 7627–7630.