Altering intracellular pH reveals the kinetic basis of intraburst gating in the CFTR Cl⁻ channel

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KEY POINTS SUMMARY

- The cystic fibrosis transmembrane conductance regulator (CFTR), which is defective in the genetic disease cystic fibrosis (CF), forms a gated pathway for chloride movement regulated by intracellular ATP.

- To understand better CFTR function, we investigated the regulation of channel openings by intracellular pH.

- We found that short-lived channel closures during channel openings represent subtle changes in the structure of CFTR that are regulated by intracellular pH, in part, at ATP-binding site 1 formed by the nucleotide-binding domains.

- Our results provide a framework for future studies to understand better the regulation of channel openings, the dysfunction of CFTR in CF and the action of drugs that repair CFTR gating defects.
ABSTRACT

Cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-gated Cl− channel defective in the genetic disease cystic fibrosis (CF). The gating behaviour of CFTR is characterized by bursts of channel openings interrupted by brief, flickery closures, separated by long closures between bursts. Entry to and exit from an open burst is controlled by the interaction of ATP with two ATP-binding sites, sites 1 and 2 in CFTR. To understand better the kinetic basis of CFTR intraburst gating, we investigated the single-channel activity of human CFTR at different intracellular pH (pHi) values. When compared with the control (pHi 7.3), acidifying pHi to 6.3 or alkalinizing pHi to 8.3 and 8.8 caused small reductions in the open-time constant \( \tau_o \) of wild-type CFTR. By contrast, the fast closed-time constant \( \tau_{cf} \), which describes the short-lived closures that interrupt open bursts, was greatly increased at pH i 5.8 and 6.3. To analyse intraburst kinetics, we used linear three-state gating schemes. All data were satisfactorily modeled by the \( C_1 \leftrightarrow O \leftrightarrow C_2 \) kinetic scheme. Changing the intracellular ATP concentration was without effect on \( \tau_o \), \( \tau_{cf} \) and their responses to pH i changes. However, mutations that disrupt the interaction of ATP with ATP-binding site 1, including K464A, D572N and the CF-associated mutation G1349D all abolished the prolongation of \( \tau_{cf} \) at pHi 6.3. Taken together, our data suggest that the regulation of CFTR intraburst gating is distinct from the ATP-dependent mechanism that controls channel opening and closing. However, our data also suggest that ATP-binding site 1 modulates intraburst gating.
ABBREVIATIONS

ABC, ATP-binding cassette; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IBI, interburst interval; MBD, mean burst duration; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; $P_o$, open probability; R domain, regulatory domain.
INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel transporting Cl\(^{-}\) and HCO\(_3\)^{-} across the apical membrane of epithelial cells (Hwang & Kirk, 2013). Structurally, CFTR belongs to the ATP-binding cassette (ABC) family, but distinctively it forms a ligand-gated ion channel (Hwang & Kirk, 2013). CFTR contains two membrane-spanning domains (MSDs) that form the channel pore, two nucleotide-binding domains (NBDs) that bind ATP molecules to control channel gating, and a unique regulatory domain (R domain) that confers CFTR activation by PKA-dependent phosphorylation (Hwang & Kirk, 2013). CFTR dysfunction causes the genetic disease cystic fibrosis (CF) (Riordan et al., 1989). Because CF mutations frequently disrupt channel gating, understanding gating mechanisms in CFTR is important for deciphering the pathogenesis of CF and developing mutation-specific therapies.

The gating pattern of CFTR is characterized by bursts of openings interrupted by short-lived closures and separated by long closures between bursts (Anderson et al., 1991; Winter et al., 1994). Early studies demonstrated that the transition between long closures and bursts of openings is regulated by ATP binding and hydrolysis at the NBDs (Anderson et al., 1991; Hwang et al., 1994; Carson et al., 1995; Lansdell et al., 1998; Zeltwanger et al., 1999; Ikuma & Welsh, 2000; Vergani et al., 2003). Later studies revealed that the two NBDs form a head-to-tail dimer with an interface containing two ATP-binding sites, site 1 and site 2 (Lewis et al., 2004; Vergani et al., 2005). Evidence shows that the turnover rate of ATP at site 1 is less than that at site 2 in CFTR gating (Tsai et al., 2009; Tsai et al., 2010), because site 1 exhibits reduced or absent ATP hydrolytic activity (Aleksandrov et al., 2002; Lewis et al., 2004; Kidd et al., 2004). With ATP stabilizing the two NBDs at site 1, NBD dimerization by ATP binding at site 2 powers CFTR opening (Vergani et al., 2005).
Recent gating models suggest that site 2 cyclically binds and hydrolyzes ATP to drive channel gating (Aleksandrov et al., 2002; Vergani et al., 2005; Csanady et al., 2010) at a slow pace, about once per second at room temperature (Li et al., 1996). However, CFTR closing may not be strictly coupled to ATP hydrolysis as at least two open states are found to occur during channel opening (Hennager et al., 2001; Jih et al., 2012). In addition, significant structural rearrangement at site 1 may accompany the CFTR gating cycle induced by ATP binding and hydrolysis at site 2 (Csanady et al., 2013), suggesting cross talk between sites 1 and 2 to regulate channel gating.

Most studies of CFTR gating have focused on transitions between the long closures and bursts of channel opening. The gating kinetics of short-lived channel closures within a burst have received less attention. Previous studies have attributed intraburst closures to recording noise, channel pore blockage by buffer ions such as HEPES (Dalemans et al., 1991; Haws et al., 1992; Tabcharani et al., 1997; Zhou et al., 2001) and MOPS (Ishihara & Welsh, 1997) or intrinsic conformational changes in CFTR itself (Ishihara & Welsh, 1997; Cai et al., 2003). Intriguingly, the intraburst activity of CFTR resembles the gating behaviour of ligand-gated channels such as cyclic nucleotide-gated ion channels (Sunderman & Zagotta, 1999). Moreover, intraburst closures are sensitive to membrane voltage (Cai et al., 2003) and temperature (Ishihara & Welsh, 1997) and differ between species (Lansdell et al., 1998; Cai et al., 2015). These data suggest that sequential openings and closings within a burst might be associated with kinetic shifts in CFTR conformation and modulated by physiological stimuli.

To test this hypothesis, we studied the single-channel kinetics of wild-type and mutant CFTR. Because intracellular pH (pHi) alters CFTR gating (Chen et al., 2009), we first tested whether intraburst activity is sensitive to different pHi solutions. To investigate the underlying regulatory mechanisms for CFTR intraburst activity and its pHi sensitivity, we
tested several well-known mutants, including the CF mutations ΔF508, G551D and G1349D. Our data reveal that intraburst activity in CFTR is operated by an ATP-independent gating mechanism, but associated with the interaction of ATP at site 1. Our findings suggest that channel openings occur when CFTR enters the ATP-driven bursting state, wherein an ATP-independent mechanism closes the channel gate intermittently to generate short-lived intraburst closures.

METHODS

Cells and CFTR expression

Experimental details have been described previously (Chen et al., 2009). Briefly, we used mammalian cells heterologously expressing human CFTR constructs. HeLa cells were used to transiently express K464A- and D572N-CFTR by the vaccinia virus/bacteriophage T7 hybrid expression system (Rich et al., 1990) and wild-type and ΔF508-CFTR by plasmid transfection with Lipofectamine 2000 (Invitrogen) in some experiments. Other CFTR variants were stably expressed as follows: wild-type, ΔF508-, ΔRS660A- and G1349D-CFTR in mouse mammary epithelial (C127) cells; G551D-CFTR in Fischer rat thyroid (FRT) cells and K1250M-CFTR in NIH 3T3 cells. The single-channel behaviour of wild-type human CFTR in different mammalian cells is equivalent (Chen et al., 2009).

Electrophysiology

CFTR currents in excised inside-out membrane patches were recorded using Axopatch 200A or 200B patch-clamp amplifiers and analyzed with pCLAMP software (all from Molecular Devices, Union City, CA, USA) as described previously (Sheppard & Robinson, 1997; Chen et al., 2009). The pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine (NMDG), 140 aspartic acid, 5 CaCl₂, 2 MgSO₄ and 10 N-
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[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.3 with Tris ([Cl\textsuperscript{-}], 10 mM). The control bath (intracellular) solution contained (mM): 140 NMDG, 3 MgCl\textsubscript{2}, 1 CsEGTA, 5 Trizma base and 5 Bis-Tris, pH 7.3 with HCl, ([Cl\textsuperscript{-}], 147 mM; free [Ca\textsuperscript{2+}], <10\textsuperscript{-8} M) at 37 °C. To ensure identical Cl\textsuperscript{-} concentrations, pH solutions were first titrated to pH 7.3 with HCl before titrating with H\textsubscript{2}SO\textsubscript{4} to acidic pH or Tris to alkaline pH values.

CFTR channels in excised inside-out membrane patches were activated by adding PKA (75 nM) and ATP (1 mM) to the bath solution. Channel activity was maintained by adding fresh PKA (75 nM) and ATP (0.3 or 1 mM) at the start of each intervention. Membrane voltage was clamped at –50 mV. Experimental protocols and conditions were performed as described previously (Chen et al., 2009). To augment the activity of CFTR mutants in the NBDs, we used ATP at 1 mM, whereas wild-type and ΔRS660A-CFTR were routinely studied using ATP at 0.3 mM. Most CFTR single-channel currents were initially recorded on digital audiotape (Biologic Scientific Instruments, model DTR-1204; Intracel Ltd., Royston, UK) at a bandwidth of 10 kHz, while some were directly digitized and stored in the computer. For digitization, recordings were filtered with an 8-pole Bessel filter (model 902LPF2 or 900; Frequency Devices, Inc., Ottawa, IL, USA) at a corner frequency (f\textsubscript{c}) of 500 Hz and acquired using a Digidata 1200 or 1440 interface (Molecular Devices) and pCLAMP software at the sampling rate of 5 kHz. For the purpose of illustration, current recordings were filtered at 500 Hz and digitized at 1 kHz.

The number of active channels in a membrane patch was determined by the maximum number of recorded channels that opened simultaneously at any one time during the entire experiment. To obtain burst durations, the channel bursts formed by only one active channel were measured. For open probability (P\textsubscript{o}) and burst analysis, event lists of open- and closed-times were created using pCLAMP software with a half-amplitude crossing criterion.
Transitions ≤ 1 ms were excluded from event lists (eight-pole Bessel filter rise time ($T_{10-90}$) ~0.73 ms at $f_c = 500$ Hz).

Single-channel open- and closed-time histograms were created using logarithmic x-axes with 10 bins per decade. Using the maximum likelihood method, open- and closed-time histograms were fitted with one- or two-component exponential functions, respectively. The mean values of exponential functions were used to derive open- and closed-time constants. $P_o$ was calculated from open and closed times. To measure mean burst duration (MBD), interburst interval (IBI) and $P_o$ within a burst ($P_o$-burst), burst analysis was performed using recordings from membrane patches that contained 1-4 active channels. The delimiter time ($t_c$) that separates interburst closures from intraburst closures was determined from the point of intersection between the two exponential curves fitting the fast and slow populations of channel closures in the closed-time histogram, as described previously (Carson et al., 1995).

Event lists and $t_c$ values were used to derive MBD and $P_o$-burst with pCLAMP software. Then, IBI was calculated using Equation 1:

$$P_o = \frac{\text{MBD} \times P_{o\text{-burst}}}{\text{MBD} + \text{IBI}}$$  \hspace{1cm} \text{Equation (Eq.) 1}

To develop kinetic gating schemes, we used QuB software (www.qub.buffalo.edu; Qin et al., 1997) with maximum likelihood analysis (Cai et al., 2003), excluding transitions ≤ 1 ms. Only data from membrane patches that contained a single active channel were used for kinetic modeling.

**Reagents and chemicals**

With the exception of PKA purified from bovine heart (Promega, Southampton, UK and Calbiochem/Merck Millipore, Darmstadt, Germany), chemicals were purchased from the Sigma-Aldrich Company Ltd. (Gillingham, UK). Stock solutions of ATP were prepared fresh before each experiment.
Statistics

One-way ANOVA and paired Student’s t-test were used to analyze sets of data. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Acidic and alkaline intracellular solutions alter the intraburst activity of CFTR

To investigate the intraburst activity of the CFTR Cl$^-$ channel, we studied the single-channel activity of wild-type human CFTR at different intracellular pH ($p_{Hi}$) values from $p_{Hi}$ 5.8 to 8.8 (Fig. 1A and B). The gating pattern of CFTR is characterized by bursts of openings, separated by long closures and interrupted by short-lived closures within bursts (Fig. 1). Figures 1 and 2 and Table 1 demonstrate that $p_{Hi}$ had complex effects on CFTR channel gating. Consistent with our previous results (Chen et al., 2009), the interburst activity of CFTR measured by open probability ($P_o$), mean burst duration (MBD) and interburst interval (IBI) had diverse responses to different $p_{Hi}$ solutions (Fig. 1 and Table 1). For example, at $p_{Hi}$ 6.3 $P_o$ increased 1.5-fold because MBD increased 2.7-fold and IBI decreased 0.7-fold. By contrast, at $p_{Hi}$ 8.3 $P_o$ decreased 0.7-fold because MBD decreased 0.6-fold and IBI increased 1.3-fold.

To examine whether intraburst openings and closings are sensitive to $p_{Hi}$ changes, we measured their dwell times using the open- and closed-time histograms (Fig. 2A-D). Our data demonstrate that the open-time constant ($\tau_o$) was decreased ~0.8-fold at $p_{Hi}$ 6.3, $p_{Hi}$ 8.3 and $p_{Hi}$ 8.8, but unchanged at $p_{Hi}$ 5.8 (Fig. 2A, C and E). Of note, the fast closed-time constant ($\tau_{cf}$), representing the population of short-lived intraburst closures in the closed-time histogram (Fig. 2B and D) was increased 1.6-fold at $p_{Hi}$ 6.3 and 1.5-fold at $p_{Hi}$ 5.8, but was
un alters at alkaline pHi (Fig. 2B, D and F). Thus, the data suggest that CFTR intraburst gating described by $\tau_o$ and $\tau_{cf}$ is sensitive to pHi changes.

Consistent with the analysis of bursts (Table 1), pHi had complex effects on the slow closed-time constant ($\tau_{cs}$), representing the population of long interburst closures: $\tau_{cs}$ decreased 0.6-fold at pHi 6.3, increased 3.7-fold at pHi 5.8, increased 1.3-fold at pHi 8.3, but was unaltered at pHi 8.8 (Fig. 2B, D and G). Moreover, the diverse responses of $\tau_{cs}$ and $\tau_{cf}$ to different pHi solutions suggest that the long interburst closures and short-lived intraburst closures might be regulated by distinct mechanisms.

Buffers are without effect on the intraburst activity of the CFTR Cl$^-\text{channel}$

A caveat for analyzing CFTR intraburst gating is that short-lived closures might result from blockage of the channel pore by buffer ions, such as HEPES (Dalemans et al., 1991; Haws et al., 1992; Tabcharani et al., 1997; Zhou et al., 2001), TES (Tabcharani et al., 1997) and MOPS (Ishihara & Welsh, 1997). To address this possibility, we tested whether increasing the concentration of the buffers Trizma or Bis-Tris three-fold in the intracellular solution might alter CFTR intraburst gating (Fig. 3A-C). The data demonstrate that increasing the concentrations of either Trizma or Bis-Tris did not affect MBD, $\tau_o$ and $\tau_{cf}$ of wild-type CFTR (Fig. 3A-C). Similarly, using intracellular solutions with a different buffer, TES (10 mM), or the same Trizma buffer, but at a very low concentration (0.1 mM) had little or no effect on MBD, $\tau_o$, $\tau_{cf}$ and $\tau_{cs}$ (Fig. 3D-G). Thus, the data suggest that intraburst closures were unlikely to be caused by buffer-generated blockage of the CFTR channel pore. Since the intraburst closures (Fig. 1) were also distinct from biphasic recording noise, the data suggest that openings and closings within a burst might represent stable and integral conformational states during CFTR gating.
**Kinetic modeling of CFTR gating at different pH**

Complex cyclic gating models have been developed to describe CFTR channel gating by ATP binding and hydrolysis at the NBDs (Tsai et al., 2010; Jih et al., 2012; Csanady et al., 2013). However, to investigate intraburst gating of CFTR at different pH, we utilized the simple linear three-state kinetic schemes $C_1 \leftrightarrow O \leftrightarrow C_2$ and $C_1 \leftrightarrow C_2 \leftrightarrow O$ to analyze transitions between the long closed state $C_1$, short-lived closed state $C_2$, and open state $O$ (Fig. 4A and F) (Winter et al., 1994; Cai et al., 2003). In both kinetic schemes, the rate constants $\beta$, $\alpha$, and $\alpha_2$ describe the transition rates between three gating states and bursts of channel opening are modeled by the transitions $O \leftrightarrow C_2$ or $C_2 \leftrightarrow O$ (Fig. 4A and F, see dashed boxes).

In the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme, the relationships between the rate constants and other kinetic parameters can be described by Equation 2 (Colquhoun & Hawkes, 1982; Sakmann & Trube, 1984):

$$\text{IBI} = \frac{1}{\beta_1} ; \text{MBD} = \frac{(\beta_2 + \alpha_2)}{\alpha_1 \alpha_2} ; \tau_{cb} = \frac{1}{\alpha_2} ; \tau_o = \frac{1}{\alpha_1 + \beta_2} \quad \text{Eq. 2}$$

In the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme, an increase in the rate constant $\beta_1$ at pH 6.3 (Fig. 4B) decreased IBI (Table 1) and $\tau_{cs}$ (Fig. 2G), whereas reductions in $\beta_1$ at pH 8.3 and 5.8 (Fig. 4B) enhanced IBI and $\tau_{cs}$ at these pH values (Table 1 and Fig. 2G). In addition, decreases in $\alpha_1$ prolonged MBD at acidic pH 5.8 and 6.3 (Fig. 4D and Table 1), whereas increases in $\alpha_1$ shortened MBD at alkaline pH 8.3 and 8.8 (Fig. 4D and Table 1). For CFTR intraburst gating, the increased $\beta_2$ rate constant at pH 6.3 (Fig. 4C) might cause a small decrease in $\tau_o$ (Fig. 2E) as $\tau_o = \frac{1}{(\alpha_1 + \beta_2)}$ (Eq. 2). Following this equation, the enhanced $\alpha_1$ rate constant at alkaline pH (Fig. 4D) might also cause a small reduction in $\tau_o$ (Fig. 2E). Moreover, the marked increase in $\tau_{cb}$ at acidic pH 6.3 and 5.8 (Fig. 2F) might be caused by large reductions in the $\alpha_2$ rate constant (Fig. 4E) as $\tau_{cb} = \frac{1}{\alpha_2}$ (Eq. 2). Taken together, these data suggest that the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme adequately accounts for the pH-sensitivity of CFTR interburst and intraburst gating.
Because the $C_1 \leftrightarrow O \leftrightarrow C_2$ and $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic schemes are mathematically equivalent (Colquhoun & Hawkes, 1982; Sakmann & Trube, 1984; Kienker, 1989), we next modeled CFTR gating using the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme. In the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme, the relationships between rate constants and kinetic parameters can be described by Equation 3 (Colquhoun & Hawkes, 1982; Sakmann & Trube, 1984):

$$\text{IBI} = \tau_c = \frac{1}{\beta_1} \left[ 1 + \left( \frac{\alpha_1}{\beta_2} \right) \right] + \left( \frac{1}{\beta_2} \right); \text{MBD} = \frac{(\beta_2 + \alpha_1)^2 + \beta_2 \alpha_2}{(\beta_2 + \alpha_1)\alpha_1 \alpha_2}; \tau_{cf} = \frac{1}{\alpha_1 + \beta_2}; \tau_o = \frac{1}{\alpha_2} \quad \text{Eq. 3}$$

Consistent with the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme (Fig. 4B and D), alterations in $\beta_1$ and $\alpha_1$ in the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme (Fig. 4G and I) accounted for the changes in IBI and MBD, respectively, at different pH$_i$ (Eq. 3 and Table 1). However, the rate constants $\beta_2$ and $\alpha_2$, which describe intraburst gating in this scheme were little altered at different pH$_i$ (Fig. 4H and J). Instead, the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme indicated that alterations in $\tau_{cf}$ at different pH$_i$ (Fig. 2F) were caused by the corresponding changes in the rate constant $\alpha_1$ (Fig. 4I) as $\tau_{cf} = 1/(\alpha_1 + \beta_2)$ (Eq. 3). Moreover, the small decreases in $\tau_o$ at pH$_i$ 6.3, 8.3 and 8.8 (Fig. 2E) were not well simulated by the rate constant $\alpha_2$ in the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme (Fig. 4J).

To further compare the modeling results of these two kinetic schemes (Fig. 4) with the measured data in Figure 2 and Table 1, we derived the kinetic parameters $P_0$, $P_o$ within a burst ($P_{o-burst}$), MBD, IBI, $\tau_o$, $\tau_{cf}$ and $\tau_{cs}$ (Tables 2 and 3) using the rate constant data at acidic pH$_i$ 6.3 and 5.8 (Fig. 4). At pH$_i$ 6.3 and 5.8, we observed large changes in CFTR intraburst gating (Fig. 1 and 2). Tables 2 and 3 demonstrate that the kinetic parameters derived using both schemes at pH$_i$ 6.3 and 5.8 were comparable to our measured data. However, the $\tau_o$ value derived by the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme was not significantly decreased at pH$_i$ 6.3 compared to that at pH$_i$ 7.3 (Table 3), consistent with the modeling results (Fig. 4J). To interpret these data, we speculate that although mathematically the two kinetic schemes generate similar modeling results, the kinetic relationship between the three gating states
might prevent the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme from adequately modeling pH$_i$-sensitive changes in CFTR intraburst gating.

As the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme consistently well described pH$_i$-sensitive intraburst gating of CFTR, we selected this kinetic scheme to analyse data acquired in subsequent experiments. Because CFTR gating is ATP-dependent (Hwang & Kirk, 2013), we began by testing whether the pH$_i$-sensitive intraburst activity of CFTR is regulated by the ATP concentration. For these experiments, we studied channel gating at pH$_i$ 6.3, because it induced significant changes in CFTR intraburst activity (Figs. 1 and 2).

**ATP-dependence of acid-sensitive intraburst gating**

Numerous studies have demonstrated that the opening rate of CFTR or the rate constant $\beta_1$ in the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme is ATP-dependent (Winter et al., 1994; Venglarik et al., 1994; Li et al., 1996; Zeltwanger et al., 1999; Cai & Sheppard, 2002; Vergani et al., 2003). Previous studies (Winter et al., 1994; Li et al., 1996; Lansdell et al., 1998) also demonstrate that $\tau_o$ and $\tau_{cf}$ are independent of the intracellular ATP concentration. Nevertheless, some data raise the possibility that intraburst gating might be ATP-dependent (Zeltwanger et al., 1999; Cai & Sheppard, 2002; Cai et al., 2015).

Figure 5 demonstrates the effects of different ATP concentrations on CFTR gating at pH$_i$ 6.3 and 7.3. At both pH$_i$ 7.3 and 6.3, $\tau_{cs}$ was markedly decreased from 0.03 to 0.3 mM ATP and further reduced from 0.3 to 1 mM ATP (Fig. 5B). Interestingly, $\tau_{cs}$ at pH$_i$ 6.3 was smaller than that at pH$_i$ 7.3 in the presence of ATP at 0.3 and 1 mM, but not at 0.03 mM, (Fig. 5B), suggesting that when the ATP concentration is very low, the collision frequency of ATP molecules with CFTR becomes the rate-limiting factor for channel opening. When compared to values at pH$_i$ 7.3, the effects of pH$_i$ 6.3 on $\tau_o$ and $\tau_{cf}$ were similar among all three ATP concentrations tested (Fig. 5D-E). Interestingly, values of MBD were significantly prolonged.
at pH\textsubscript{i} 6.3 compared to those at pH\textsubscript{i} 7.3, particularly at 0.3 and 1 mM ATP (Fig. 5C), suggesting that the ATP collision rate might affect the stability of CFTR’s bursting state at acidic pH\textsubscript{i}. Consistent with the $\tau_{cs}$ changes (Fig. 5B), only the rate constant $\beta_1$ in the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme was sensitive to the ATP concentration (Fig. 5F-I).

Conversely, the kinetic parameters for CFTR intraburst gating including the time constants $\tau_o$ and $\tau_{cf}$ (Fig. 5D and E) and rate constants $\beta_2$ and $\alpha_2$ (Fig. 5H and I) were insensitive to the ATP concentration at both pH\textsubscript{i} 7.3 and 6.3, suggesting that intraburst openings and closings are not regulated by ATP. However, it is uncertain whether the intraburst closings might represent the intermediate closed state when CFTR has already bound ATP prior to channel opening (Haws \textit{et al.}, 1992; Venglarik \textit{et al.}, 1994; Zeltwanger \textit{et al.}, 1999). Using the two linear kinetic schemes (Fig. 4A and F), we examined this possibility by analyzing chemical kinetics (see Appendix A) to mathematically derive the relationship between the $P_0$ of CFTR and the ATP concentration, which is best described by the Michaelis-Menten equation (Anderson \textit{et al.}, 1991; Venglarik \textit{et al.}, 1994; Zeltwanger \textit{et al.}, 1999; Cai & Sheppard, 2002; Vergani \textit{et al.}, 2003; Scott-Ward \textit{et al.}, 2007; Chen \textit{et al.}, 2009). The modeling results show that both kinetic models required an intermediate closed state $C_1'$ between the long closed state $C_1$ and the bursting state to derive a Michaelis-Menten-like relationship (e.g. the $C_1 \leftrightarrow C_1' \leftrightarrow O \leftrightarrow C_2$ kinetic scheme in Appendix A, Eq. A7 for the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme). Therefore, the short-lived $C_2$ state is unlikely to represent an ATP-bound intermediate closed state in CFTR gating. These data also suggest that CFTR intraburst gating might be controlled by a gating mechanism that follows ATP binding to CFTR.

Next, we explored whether the interaction of ATP molecules with ATP-binding sites 1 and 2 affects the intraburst activity of CFTR. For these experiments, we studied several CFTR mutants in the NBDs that disturb ATP binding and hydrolysis.
Role of the R domain and ATP-binding sites in CFTR intraburst gating

To disrupt ATP-dependent regulation of CFTR channel gating, we selected four CFTR variants (Fig. 6 and 7): (i) ΔRS660A-CFTR, which deletes a large part of the R domain and likely impacts the function of both ATP-binding sites (Rich et al., 1991; Winter & Welsh, 1997; Mense et al., 2006); (ii) K1250M-CFTR, which impairs ATP binding and hydrolysis at site 2 (Carson et al., 1995; Vergani et al., 2003; Vergani et al., 2005); (iii) K464A-CFTR, which perturbs ATP binding at site 1 (Carson et al., 1995; Vergani et al., 2003) and (iv) D572N-CFTR, which attenuates Mg\(^{2+}\) binding at site 1 (Vergani et al., 2003).

Figure 6 shows representative recordings (Fig. 6A), \(\tau_0\) and \(\tau_{cf}\) data (Fig. 6B and C) of ΔRS660A-CFTR tested at pH\(_i\) 7.3 and pH\(_i\) 6.3 in the presence of 0.3 mM ATP. Compared to that of wild-type CFTR, \(\tau_0\) was reduced, but \(\tau_{cf}\) was enhanced in ΔRS660A-CFTR at pH\(_i\) 7.3 (see # symbols, Fig. 6B and C). However, similar to that in wild-type CFTR, pH\(_i\) 6.3 decreased \(\tau_0\) but increased \(\tau_{cf}\) of ΔRS660A-CFTR (Fig. 6B and C). Moreover, Figure 7 shows representative recordings, \(\tau_0\) and \(\tau_{cf}\) data of CFTR NBD mutants at pH\(_i\) 7.3 and pH\(_i\) 6.3 in the presence of 1 mM ATP. When compared with values for wild-type CFTR, the \(\tau_0\) of K464A-CFTR at pH\(_i\) 7.3 was decreased (Fig. 7B), whereas the K1250M mutation appeared to decrease \(\tau_0\) (\(P = 0.12\)), but increase \(\tau_{cf}\) (\(P = 0.08\)) at pH\(_i\) 7.3 (Fig. 7B and C). Interestingly, the \(\tau_0\) reduction by pH\(_i\) 6.3 in wild-type CFTR was abolished by the NBD mutations K1250M, K464A and D572N (Fig. 7B), whereas the \(\tau_{cf}\) elongation by pH\(_i\) 6.3 was absent in the mutants K464A- and D572N-CFTR (Fig. 7C). We interpret these results to suggest that the R domain and ATP-binding sites might contribute to the regulation of CFTR intraburst gating. The data also suggest that both ATP-binding sites might contribute to the reduction in \(\tau_0\) at pH\(_i\) 6.3, whereas only site 1 might mediate the prolongation of \(\tau_{cf}\) at pH\(_i\) 6.3.
The CF mutation G1349D greatly disturbs CFTR intraburst gating

To further investigate the roles of ATP-binding sites 1 and 2 and learn whether CF mutations perturb intraburst gating, we studied the CF mutations, ΔF508, G551D and G1349D at 1 mM ATP (Fig. 8). Located on the surface of NBD1, ΔF508 not only perturbs communication between the NBDs and MSDs (Serohipos et al., 2008; Monnon et al., 2008; Dong et al., 2011), but also destabilizes the NBD1:NBD2 dimer (Jih et al., 2011). By contrast, G551D and G1349D affect equivalent residues in the LSGGQ motifs in NBD1 and NBD2, which contribute to site 2 and site 1, respectively (Lewis et al., 2004; Cai et al., 2006; Bompadre et al., 2007). Both mutations perturb severely CFTR channel gating, with G551D rendering CFTR gating ATP-independent (Bompadre et al., 2007).

Consistent with previous studies (Cai et al., 2006), G551D and G1349D not only greatly prolonged IBI, but noticeably reduced the MBD of CFTR (Fig. 8A and B). Interestingly, pH_i 6.3 only enhanced the MBD of ΔF508-CFTR among the three CF mutants studied (Fig. 8B). However, the fold change of MBD in ΔF508-CFTR was less than that of wild-type CFTR (MBD_pH_i 6.3/MBD_pH_i 7.3: ΔF508-CFTR, 1.8 ± 0.2; wild-type CFTR, 2.8 ± 0.2; N = 6, P < 0.05, one-way ANOVA; Fig. 8B). Of note, at pH_i 6.3 the MBD of G1349D-CFTR was significantly reduced (Fig. 8A and B).

For CFTR intraburst gating, only G1349D caused large reductions in both τ_o and τ_cbf at pH_i 7.3 (Fig. 8C and D). Like wild-type CFTR, the τ_o of all three CF mutants at pH_i 6.3 was shorter than that at pH_i 7.3 (Fig. 8C, P = 0.061 for G551D). However, only G551D and G1349D abolished the prolongation of τ_cbf at pH_i 6.3 (Fig. 8D). Moreover, the MBD/τ_o ratio, which is used to estimate the average number of channel openings within a burst of openings was unchanged in ΔF508-CFTR compared to that of wild-type CFTR (Fig. 8E). Conversely, the MBD/τ_o ratio at both pH_i 7.3 and pH_i 6.3 was reduced to 1.7 in G551D-CFTR and 1.3 in G1349D-CFTR compared to 2.8 at pH_i 7.3 for wild-type CFTR (see # symbols, Fig. 8E).
These data suggest that bursts of channel openings were very difficult to form in G1349D-CFTR such that each burst often only appeared to contain a single opening (Fig. 8A). Taken together, our data indicate that CFTR intraburst gating and its pH-sensitivity were altered slightly by ΔF508, moderately by G551D, but severely by G1349D.

**DISCUSSION**

This study aimed to investigate intraburst gating in the CFTR Cl⁻ channel by exploiting the effects of pHᵢ on gating kinetics. Our data reveal that intraburst openings and closings are integral gating events. The $C₁ \leftrightarrow O \leftrightarrow C₂$ kinetic scheme adequately simulated CFTR intraburst gating at different pHᵢ values. Mutations in ATP-binding site 1, particularly the CF mutation G1349D, had greater impact on intraburst gating than those in ATP-binding site 2.

**Nature of the intraburst closures**

When recording the single-channel activity of CFTR, we and other groups (e.g. Ishihara & Welsh, 1997; Tabcharani et al., 1997; Vergani et al., 2005; Fuller et al., 2005; Bompadre et al., 2007) consistently observed burst-like openings when the channel is open. Most studies attribute this bursting behaviour of CFTR to the brief and intermittent intraburst closures, which interrupt channel openings. It is proposed that these intraburst closures are caused by blockage of the CFTR pore by buffer ions (Ishihara & Welsh, 1997; Tabcharani et al., 1997; Zhou et al., 2001) or unknown intrinsic conformational movements (Ishihara & Welsh, 1997; Cai et al., 2003). Studies using open-channel blockers of CFTR have demonstrated that fast-speed channel blockers with low binding affinity (e.g. niflumic acid, Scott-Ward et al., 2004) only intermittently and partially obstruct Cl⁻ flow through the channel pore. Conversely, intermediate-speed channel blockers with high binding affinity (e.g. glibenclamide, Sheppard & Robinson, 1997) cause full blockage of CFTR single-channel currents. The buffer ion best
known to block the CFTR pore is MOPS, which shows fast and intermediate-speed blocking behaviour at 10 mM (Ishihara & Welsh, 1997). These studies together with the present data argue that the millimolar concentrations of buffer ions in our intracellular solutions are unlikely to cause open-channel block of the CFTR pore when the channel is open. Recording noise, often seen as biphasic spikes, are unlikely to be the reason for intraburst closures. Interestingly, the short-lived closures are sensitive to the membrane voltage (Zhou et al., 2001; Cai et al., 2003). Therefore, we speculate that part of the conformational changes that underlie CFTR intraburst gating occur within the MSDs.

Control of the intraburst activity in CFTR gating

NBD dimerization and dissociation induced by cycles of ATP binding and hydrolysis at binding site 2 forms the basic gating mechanism for interburst gating of the CFTR Cl\(^{-}\) channel (Vergani et al., 2005; Scott-Ward et al., 2007; Tsai et al., 2010; Csanady et al., 2010; Jih et al., 2012). Interestingly, our study found that the effects of pH\(\text{II}\), ATP and mutations on intraburst gating (\(\tau_0\) and \(\tau_{cf}\)) of CFTR were mismatched to their effects on interburst gating such as \(\tau_{cs}\), IBI and MBD. These findings suggest the presence of a second gating mechanism that controls CFTR intraburst gating. We propose that once CFTR enters the open state during ATP-dependent gating cycles, the second gating mechanism intermittently closes the channel pore thereby generating fast and short-lived intraburst closures.

Several lines of evidence support the presence of a second gating mechanism governing the intraburst activity of CFTR. First, previous work demonstrates that disrupting salt bridges in the MSDs alters the intraburst activity of CFTR (Cotten & Welsh, 1999). Mutations on extracellular loop 1 in MSDs could destabilize the burst duration of CFTR (Sheppard et al., 1993; Cui et al., 2014; Infield et al., 2016). Second, studies of CFTR homologues have identified differences in intraburst gating (Lansdell et al., 1998; Scott-Ward
et al., 2007). Differences in the MSDs are likely responsible for the peculiar patterns of intraburst gating between murine and human CFTR (Scott-Ward et al., 2007). Similarly, ovine CFTR exhibits differences in intraburst gating to human CFTR characterized by shorter $\tau_{cf}$, but longer $\tau_o$ than human CFTR (Cai et al., 2015). Finally, while studying the permeation of $[\text{Au(CN)}_2]^{-1}$ through the channel pore of cysless-CFTR, a gate movement within a defined section of the MSDs encompassing residues 338-341 in transmembrane segment 6 was discovered recently (Gao & Hwang, 2015). These data suggest that the gating mechanism which controls the intraburst activity of CFTR varies among different species and is possibly associated with conformational changes in the MSDs.

**pH$_i$ sensitivity of CFTR intraburst gating**

Interburst closures demonstrate a different sensitivity to pH$_i$ changes than intraburst closures. Our previous work suggest that ATP-binding site 2 determines the pH$_i$ sensitivity of MBD and IBI in CFTR gating (Chen et al., 2009). Because G1349D-CFTR may have modest ATP function at site 2 (Cai et al., 2006; Bompadre et al., 2007), large alterations in its intraburst activity and pH$_i$ sensitivity are plausibly caused by ATP dysfunction at binding site 1. The site 1 mutations K464A and D572N, which prevented the $\tau_{cf}$ prolongation at pH$_i$ 6.3 may also have normal ATP function at site 2 (Vergani et al., 2003). Therefore, our data suggest that ATP-binding site 1 plays a major role in sensing acidic pH$_i$ during CFTR intraburst gating.

Several studies demonstrate that ATP binding at site 1 regulates CFTR gating by acting like a ligand. At site 1, ATP has a higher binding affinity than site 2 (Howell et al., 2000), but exhibits a low turnover rate (i.e. over ten minutes) in biochemical studies (Aleksandrov et al., 2002; Basso et al., 2003) and shows reduced or no hydrolytic activity (Aleksandrov et al., 2002; Lewis et al., 2004; Kidd et al., 2004). Conversely, patch-clamp studies indicate that ATP turnover at site 1 might be less than a minute or a few seconds (Tsai
CFTR intraburst gating

et al., 2009; Tsai et al., 2010), suggesting that ATP stability at binding site 1 during active
CFTR gating cycles might be less than that in the biochemical studies (Aleksandrov et al.,
2002; Basso et al., 2003). Consistent with this idea, Csanady et al. (2013) found that site 1
undergoes significant structural rearrangements during channel opening. Thus, CFTR
intraburst gating might resemble that of the cyclic nucleotide-gated channels (Sunderman &
Zagotta, 1999), in which unstable binding of the ligand causes dynamic conformational
rearrangements that generate the intermittent intraburst closures. Moreover, amino acid
residues such as H620, H667, C469 and C491 around the K464 and D572N residues at ATP-
binding site 1 might be candidates for sensing pH₄ changes. Future studies should explore
their potential role in CFTR intraburst gating.

By contrast, whether CFTR intraburst gating requires normal ATP function at site 2
is uncertain. This query is raised because the G551D mutation greatly impairs the ATP-
dependence of channel gating at site 2 (Cai et al., 2006; Bompadre et al., 2007), but it only
showed mild effects on CFTR intraburst gating. Intriguingly, G551D-CFTR still responds to
gating potentiators, such as genistein and phloxine B (Illek et al., 1999; Cai et al., 2006),
which enhance CFTR activity possibly by restoring NBD dimerization around site 2 (Cai et
al., 2006; Zegarra-Moran et al., 2007). Therefore, we speculate that in our recording
condition, sporadic ATP binding or NBD dimerization by an unknown ATP-independent
mechanism at site 2 might eventually transform G551D-CFTR into the bursting state, which
is required for initiating intraburst gating by a second gating mechanism. Similarly,
mutations that greatly prolong the channel opening rate such as K1250M (Carson et al.,
1995), G551D (Cai et al., 2006) and ΔF508 (Dalemans et al., 1991) only mildly or slightly
affected CFTR intraburst gating.

In conclusion, our study characterized the kinetic basis of intraburst gating in CFTR
by changing different pH₄ solutions. The data suggest that a separate gating mechanism
operating together with the ATP-driven NBD dimerization model (Vergani et al., 2005; Hwang & Kirk, 2013) is required for CFTR intraburst gating. While highlighting the complexity of CFTR gating, this study leaves some unresolved aspects of intraburst gating to future studies. For example, the mechanism that generates two short-lived closed states in CFTR intraburst gating at room temperature (Ishihara & Welsh, 1997) remains unclear. Nevertheless, this work emphasizes the importance of analysing intraburst activity to understand fully the CFTR gating mechanism. We suggest that ATP-dependent channel activity in CFTR represents cycles of transitions between the long closed state and bursting state, whereas movement of a channel gate during the bursting state might be investigated by studying intraburst gating.
APPENDIX A: ATP-dependence of the C₁ ↔ O ↔ C₂ kinetic scheme

Equation A1 (Eq. A1) describes ATP-dependent CFTR gating using the C₁ ↔ O ↔ C₂ kinetic scheme:

\[
\text{CFTR (C₁)} + \text{MgATP} \xrightarrow{k₁} \text{CFTR} \cdot \text{MgATP (O)} \xrightarrow{k₂⁻¹} \text{CFTR} \cdot \text{MgATP (C₂)}
\]

where \(k₁\), \(k₁⁻¹\), \(k₂\) and \(k₂⁻¹\) are the rate constants and CFTR·MgATP represents CFTR with bound MgATP. The rate constant \(k₁\) describes the ATP-dependent opening rate of CFTR, whereas the rate constant \(k₁⁻¹\) indicates the closing rate of CFTR due to ATP hydrolysis or release. The rate constants \(k₂\) and \(k₂⁻¹\) are used to describe the static transitions of CFTR intraburst gating. Following Eq. A1, we can obtain \(P₀\) from Eq. A2:

\[
P₀ = \frac{T₀}{T_{C₁} + T_{C₂} + T₀}
\]

where the dwell times \(T_{C₁}\), \(T_{C₂}\) and \(T₀\) represent the time CFTR spends in the three kinetic states C₁, C₂ and O, respectively. Assuming that the three kinetic states remain in equilibrium, the forward and reverse reaction rates between the two connected states are the same (Eq. A3):

\[
k₁ \times (T_{C₁} \cdot [\text{MgATP}]) = k₁⁻¹ \times T₀; \quad k₂ \times T₀ = k₂⁻¹ \times T_{C₂}
\]

Therefore, \(P₀\) is derived using Eq. A2 and A3:

\[
P₀ = \frac{1}{1 + \frac{k₁⁻¹}{k₁ \cdot [\text{MgATP}]} + \frac{k₂}{k₂⁻¹}} = \frac{k₂⁻¹[\text{MgATP}]}{k₂ + k₂⁻¹[\text{MgATP}]} + \frac{k₁⁻¹ \cdot k₂⁻¹}{k₁(k₂ + k₂⁻¹)}
\]

Consequently, as \([\text{MgATP}] \to \infty\), the maximum \(P₀\) \((P_{\text{max}}) = \frac{k₂⁻¹}{k₂ + k₂⁻¹}\)

(A5)
Although Eq. A4 describes the hyperbolic relationship between [MgATP] and the \( P_o \) of CFTR similar to the Michaelis-Menten equation (see below Eq. A6 with a constant \( K \)), \( P_{omax} \) will be close to the \( P_o \) within a burst (\( P_o \)-burst, Table 2) ~ 0.9, which is higher than our previous data ~ 0.72 at pH\(_i\) 7.3 (Chen et al., 2009). A similar result was also found for the \( C_1 \leftrightarrow C_2 \leftrightarrow O \) kinetic scheme (data not shown).

\[
P_o = \frac{P_{omax}[MgATP]}{[MgATP]+K}
\]  
(Michaelis-Menten equation)  

(A6)

A possible reason for this discrepancy is that after ATP binding to CFTR, there might be an intermediate, rate limiting state prior to the ATP-dependent conformational changes that lead to channel opening (Haws et al., 1992; Venglarik et al., 1994), i.e. the conformational changes for NBD dimerization (Vergani et al., 2005) and coupling of the NBDs and MSDs (Hwang & Kirk, 2013).

To model this ATP-dependent rate-limiting step, we added an additional gating state \( C_1' \) with the rate constants \( k_0 \) and \( k_0^{-1} \) between the \( C_1 \) and \( O \) states (the \( C_1 \leftrightarrow C_1' \leftrightarrow O \leftrightarrow C_2 \) kinetic scheme, Eq. A7).

\[
\text{CFTR} (C_1) + \text{MgATP} \xleftarrow{k_0} \text{CFTR} \cdot \text{MgATP}(C_1') \xrightarrow{k_0^{-1}} \text{CFTR} \cdot \text{MgATP}(O) \xleftarrow{k_1} \text{CFTR} \cdot \text{MgATP}(C_2) \xrightarrow{k_2^{-1}} \text{CFTR} \cdot 2\text{MgATP}(C_2)
\]  
(A7)

The \( P_o \) and \( P_{omax} \) for Eq. A7 can be derived from the following equations (Eqs. A8-A11) with \( T_{C_1'} \) representing the dwell time CFTR spends in the \( C_1' \) state:

\[
P_o = \frac{T_0}{T_{C_1'} + T_{C_1'} + T_{C_2} + T_O}
\]  
(A8)

\[
k_0 \times (T_{C_1} \cdot [\text{MgATP}]) = k_0^{-1} \times T_{C_1'}; \quad k_1 \times T_{C_1'} = k_1^{-1} \times T_O; \quad k_2 \times T_O = k_2^{-1} \times T_{C_2}
\]  
(A9)
Our calculations show that the relationship between $P_0$ and $[\text{MgATP}]$ is well described by the Michaelis-Menten-like equation (Eq. A10) with $P_{\text{omax}}$ (Eq. A11) containing the $k_1$ rate constant as the rate-limiting parameter. Moreover, Eq. A11 suggests that the $P_{\text{omax}}$ of CFTR might be close to $P_{o\text{-burst}}$ only if the rate constant $k_1^{-1}$ is close to zero causing a permanent bursting state, or if the rate constant $k_1$ becomes infinite eliminating the rate-limiting step ($C_1' \rightarrow O$). By a similar approach, we also derived the $C_1 \leftrightarrow C_1' \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme from the $C_1 \leftrightarrow C_2 \leftrightarrow O$ kinetic scheme (data not shown).

Previous work (Venglarik et al., 1994) applying noise analysis to macroscopic current recordings of wild-type CFTR demonstrated that the $C_1 \leftrightarrow C_1' \leftrightarrow O$ kinetic scheme, excluding short-lived closures, well models the ATP-dependent channel activity of CFTR using the Michaelis-Menten relationship. Their modeling results (Venglarik et al., 1994) also suggest that when the $C_1' \leftrightarrow O$ transitions are rate limiting, a single population of the long closures (Fig. 2B and D) was achieved in the $C_1 \leftrightarrow C_1' \leftrightarrow O$ kinetic scheme. Interestingly, recent studies identified the presence of transient closed states in the journey from long closures to channel openings (Scott-Ward et al., 2007; Sorum et al., 2015). These transient closed states, if rate limiting, are consistent with the $C_1'$ state supporting CFTR gating with the Michaelis-Menten relationship.

Many studies have developed complex loop models with multiple substates within the $C_1 \leftrightarrow O$ transition controlled by ATP binding and hydrolysis (Vergani et al., 2005; Scott-
Ward et al., 2007; Tsai et al., 2009; Tsai et al., 2010; Csanady et al., 2010; Jih et al., 2012; Sorum et al., 2015). The transition rates between the $C_1$ and $O$ states in the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme should represent the overall transition rates through these multiple substates. Thus, we did not further investigate more complex gating schemes in this study.

Finally, to verify our approach, we calculated $P_0$ from the $C_1 \leftrightarrow O \leftrightarrow C_2$ kinetic scheme without MgATP (Eqs. A12-14).

\[
\text{CFTR (}C_1\text{)} \xrightarrow{k_1^{-1}} \text{CFTR (}O\text{)} \xrightarrow{k_2^{-1}} \text{CFTR (}C_2\text{)}
\]  \hspace{1cm} (A12)

\[
k_1 \times T_{C_1} = k_1^{-1} \times T_O; \quad k_2 \times T_O = k_2^{-1} \times T_{C_2}
\]  \hspace{1cm} (A13)

\[
P_0 = \frac{T_O}{T_O + T_{C_1} + T_{C_2}} = \frac{1}{1 + \left(\frac{k_1}{k_1^{-1}}\right) + \left(\frac{k_2}{k_2^{-1}}\right)} = \frac{k_1 \cdot k_2^{-1}}{(k_1 + k_1^{-1})(k_2 + k_2^{-1}) - k_2 \cdot k_1^{-1}}
\]  \hspace{1cm} (A14)

Our calculations derive $P_0$ (Eq. A14) in a similar way to that reported previously (Sakmann & Trube, 1984), validating our mathematical approach using chemical kinetics.
REFERENCES


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COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

JH Chen conceived and designed experiments, acquired, analysed and interpreted most data and wrote the article. W Xu acquired and analysed some data. DN Sheppard planned the experiments, contributed to data interpretation and revised the manuscript. DN Sheppard, W Xu and JH Chen all concur with the final submitted version of the manuscript and confirm that all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. Most data were acquired and analysed at the University of Bristol; some data were acquired and analysed at the University of Hong Kong.

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Table 1. Burst analysis of wild-type CFTR gating at different pH<sub>i</sub>.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>P&lt;sub&gt;o&lt;/sub&gt;</th>
<th>MBD</th>
<th>IBI</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt; 5.8</td>
<td>0.37 ± 0.01*</td>
<td>361 ± 39*</td>
<td>534 ± 41*</td>
<td>6</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt; 6.3</td>
<td>0.66 ± 0.02*</td>
<td>358 ± 37*</td>
<td>107 ± 10*</td>
<td>6</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt; 7.3 (control)</td>
<td>0.44 ± 0.01</td>
<td>134 ± 4</td>
<td>154 ± 4</td>
<td>15</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt; 8.3</td>
<td>0.31 ± 0.03*</td>
<td>85 ± 7*</td>
<td>205 ± 18*</td>
<td>8</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt; 8.8</td>
<td>0.40 ± 0.01*</td>
<td>93 ± 6*</td>
<td>144 ± 10*</td>
<td>6</td>
</tr>
</tbody>
</table>

Kinetic parameters: P<sub>o</sub>, open probability; MBD, mean burst duration; IBI, interburst interval.

Data are means ± S.E.M.; *, P < 0.05 vs. pH<sub>i</sub> 7.3 (control), one-way ANOVA. The Table includes data that were previously reported (Chen et al., 2009).
Table 2. Comparison of kinetic parameters derived using pCLAMP and QuB software.

<table>
<thead>
<tr>
<th>Model / pH</th>
<th>pH 6.3</th>
<th>pH 5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P&lt;sub&gt;o&lt;/sub&gt;</strong></td>
<td>6.3</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>P&lt;sub&gt;o-burst&lt;/sub&gt;</strong></td>
<td>6.3</td>
<td>5.8</td>
</tr>
<tr>
<td>MBD (ms)</td>
<td>6.3</td>
<td>5.8</td>
</tr>
<tr>
<td>IBI (ms)</td>
<td>6.3</td>
<td>5.8</td>
</tr>
</tbody>
</table>

**pCLAMP**

- **C<sub>1</sub>↔O↔C<sub>2</sub>**
  - 0.47±0.02 0.66±0.02* 0.95±0.01 0.87±0.02* 147±5 358±37* 153±9 107±10*
  - 0.43±0.01 0.37±0.02* 0.92±0.01 0.89±0.02* 113±3 299±19* 138±10 433±45*

- **C<sub>1</sub>↔C<sub>2</sub>↔O**
  - 0.47±0.02 0.69±0.02* 0.93±0.01 0.87±0.01* 137±5 326±21* 137±9 83±7*
  - 0.42±0.01 0.38±0.02* 0.92±0.01 0.89±0.01* 117±3 297±17* 143±9 412±44*

**P<sub>o-burst</sub>, P<sub>o</sub> within a burst.** Data are means ± S.E.M. of N observations; *, P < 0.05 vs. pH 7.3 (control), paired Student’s t-test. Data from pCLAMP software were obtained by burst analysis and time constant measurements using the event lists from CFTR single-channel recordings (see Methods for details). Data from QuB software were derived using the rate constants in the C<sub>1</sub> ↔ C<sub>2</sub> ↔ O and C<sub>1</sub> ↔ O ↔ C<sub>2</sub> kinetic schemes (Fig. 4) with Equations 2 and 3 (see text for details). Only membrane patches that contained a single CFTR Cl<sup>-</sup> channel were used for analysis. See Table 1 for other details.
**Table 3.** Comparison of time constants derived using pCLAMP and QuB software.

<table>
<thead>
<tr>
<th>Model / pH</th>
<th>pH: 6.3 (N = 6)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\tau_0) (ms)</td>
<td>(\tau_{cf}) (ms)</td>
<td>(\tau_{cs}) (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCLAMP</td>
<td>48±3</td>
<td>38±5*</td>
<td>3.1±0.2</td>
<td>4.9±0.4*</td>
<td>136±11</td>
</tr>
<tr>
<td>C(_1)↔O↔C(_2)</td>
<td>42±3</td>
<td>31±4*</td>
<td>3.2±0.2</td>
<td>4.7±0.5*</td>
<td>135±70</td>
</tr>
<tr>
<td>C(_1)↔C(_2)↔O</td>
<td>39±5</td>
<td>33±4</td>
<td>3.2±0.2</td>
<td>4.6±0.5*</td>
<td>137±90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model / pH</th>
<th>pH: 5.8 (N = 6)</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(\tau_0) (ms)</td>
<td>(\tau_{cf}) (ms)</td>
<td>(\tau_{cs}) (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCLAMP</td>
<td>46±5</td>
<td>50±7</td>
<td>3.2±0.2</td>
<td>4.4±0.2*</td>
<td>154±14</td>
</tr>
<tr>
<td>C(_1)↔O↔C(_2)</td>
<td>38±4</td>
<td>39±5</td>
<td>3.2±0.1</td>
<td>4.4±0.2*</td>
<td>138±10</td>
</tr>
<tr>
<td>C(_1)↔C(_2)↔O</td>
<td>38±4</td>
<td>39±5</td>
<td>3.3±0.1</td>
<td>4.5±0.3*</td>
<td>143±90</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. of N observations; *, \(P < 0.05\) vs. pH: 7.3 (control), paired Student’s t-test. See Figure 2 and Table 2 for other details.
FIGURES AND LEGENDS

Figure 1. Altering pH\textsubscript{i} affects the intraburst activity of wild-type CFTR. A and B, representative recordings show the single-channel activity of a wild-type CFTR channel at pH\textsubscript{i} 8.3, 7.3 and 6.3 (A) or at pH\textsubscript{i} 8.8, 7.3 and 5.8 (B) in the presence of ATP (0.3 mM) and PKA (75 nM). Dotted lines indicate where the channel was closed and downward deflections correspond to channel openings. The recordings in (A) and (B) are from separate wild-type CFTR Cl\textsuperscript{−} channels in different excised inside-out membrane patches.

Figure 2. Multiple effects of pH\textsubscript{i} on wild-type CFTR channel gating. A-B, open- (A) and closed-time (B) histograms of a wild-type CFTR channel at pH\textsubscript{i} 6.3, 7.3 and 8.3. Vertical lines show the open-time constant $\tau_0$, fast closed-time constant $\tau_{cf}$, and slow closed-time constant $\tau_{cs}$. The continuous coloured lines are the fits of one- or two-component exponential functions to the data. The dotted black lines in the closed-time histograms show the individual components of the functions. Logarithmic x-axes with 10 bins per decade were used for the dwell-time histograms. C-D, open- (C) and closed-time (D) histograms from a different wild-type CFTR channel tested at pH\textsubscript{i} 5.8, 7.3 and 8.8; other details as for (A) and (B). E-G, effects of acidic (yellow) and alkaline (green) pH\textsubscript{i} on $\tau_0$, $\tau_{cf}$ and $\tau_{cs}$. Data are means ± S.E.M. Numbers in parentheses indicate N for panels E-G; *, $P < 0.05$ vs. pH\textsubscript{i} 7.3 (control), one-way ANOVA. Error bars are smaller than symbol size except where shown.

Figure 3. Biological buffers are without effect on CFTR intraburst gating. A-C, effects on MBD, $\tau_0$ and $\tau_{cf}$ of pH\textsubscript{i} solutions containing 3-fold concentration increases in Trizma or Bis-Tris buffers. Circles joined by lines show values from individual experiments and columns are means ± S.E.M. Data are from membrane patches that contained one or two active CFTR Cl\textsuperscript{−} channels. D-G, effects on MBD, $\tau_0$, $\tau_{cf}$ and $\tau_{cs}$ of different buffer solutions. Circles show
values from individual experiments and columns are means ± S.E.M. For Trizma/Bis-Tris and TES groups, membrane patches that contained only one active CFTR Cl⁻ channel were used for analysis. Numbers in parentheses indicate N for panels A-C and D-G. Statistical differences between groups were analyzed by paired Student’s t-test (A-C) and one-way ANOVA (D-G).

**Figure 4.** Kinetic modeling of CFTR gating using linear three-state schemes. A and F, the C₁ ↔ O ↔ C₂ and the C₁ ↔ C₂ ↔ O kinetic schemes. States, C₁, C₂ and O represent two closed states and one open state, respectively, while β₁, β₂, α₁ and α₂ represent the rate constants describing transitions between the different states. States enclosed within the dashed box represent the bursting state. B-E, rate constants at acidic (yellow) and alkaline (green) pHi values for the C₁ ↔ O ↔ C₂ kinetic scheme. G-J, rate constants at acidic (yellow) and alkaline (green) pHi values for the C₁ ↔ C₂ ↔ O kinetic scheme. Data are means ± S.E.M. Numbers in parentheses indicate N for panels B-E and G-J; *, P < 0.05 vs. pHi 7.3 (control), one-way ANOVA. Error bars are smaller than symbol size except where shown.

**Figure 5.** Effects of pHi 6.3 on ATP-dependence of CFTR gating. A, representative recordings show the effects of pHi 6.3 on the single-channel activity of wild-type CFTR at 0.03 and 1 mM ATP. Dotted lines indicate the closed state and downward deflections correspond to channel openings. For representative recordings at 0.3 mM ATP, please see Figure 1A. B-I, effects of pHi 6.3 on MBD, the time constants τ₀, τ₁ and τ₂, and the rate constants β₁, β₂, α₁ and α₂ for the C₁ ↔ O ↔ C₂ kinetic scheme at the indicated ATP concentrations. Data are means ± S.E.M. Numbers in parentheses indicate N for panels B-I; *, P < 0.05 vs. pHi 7.3 (control), paired Student’s t-test; #, P < 0.05 between the indicated
groups of data, one-way ANOVA. Error bars are smaller than symbol size except where shown.

Figure 6. Role of the R domain in CFTR intraburst gating. A, representative recordings show the single-channel activity of wild-type and ΔRS660A-CFTR in the presence of 0.3 mM ATP at pH$_i$ 7.3 and pH$_i$ 6.3. B and C, the time constants $\tau_0$ (B) and $\tau_{cf}$ (C) at indicated pH$_i$ values. Data are means ± S.E.M. Numbers in parentheses indicate N for panels B and C; *, $P < 0.05$ vs. pH$_i$ 7.3 (control), paired Student’s t-test; #, $P < 0.05$ between the indicated groups of data, one-way ANOVA.

Figure 7. Regulation of CFTR intraburst gating by ATP-binding sites. A, representative recordings show the single-channel activity of the indicated CFTR mutants in the presence of 1 mM ATP at pH$_i$ 7.3 and pH$_i$ 6.3. B and C, the time constants $\tau_0$ (B) and $\tau_{cf}$ (C) of different CFTR mutants at the indicated pH$_i$ values. Data are means ± S.E.M. Numbers in parentheses indicate N for panels B and C; *, $P < 0.05$ vs. pH$_i$ 7.3 (control), paired Student’s t-test; #, $P < 0.05$ between the indicated groups of data, one-way ANOVA.

Figure 8. Intraburst gating of the CF mutants G551D-, G1349D- and ΔF508-CFTR. A, representative single-channel recordings of the indicated CF mutants in the presence of 1 mM ATP. Left traces show 10-s recordings; right the 1-s portions indicated by grey bars are shown on an expanded time scale. Note that the number of active channels in the G551D- and G1349D-CFTR traces is unknown. For representative control recordings of wild-type CFTR at pH$_i$ 7.3 and 6.3, please see Figure 7A. B-E, MBD, $\tau_0$, $\tau_{cf}$ and the MBD/$\tau_0$ ratio of CF mutants at pH$_i$ 7.3 and pH$_i$ 6.3. Data are means ± S.E.M. Numbers in parentheses
indicate N for panels B-E; *, $P < 0.05$ vs. pH 7.3 (control), paired Student’s t-test; #, $P < 0.05$ between the indicated groups of data, one-way ANOVA.
A

\[
pH \_i \ \begin{align*} &8.3 & 0.3 \text{ mM ATP} \\
&7.3 \\
&6.3 \end{align*}
\]

B

\[
pH \_i \ \begin{align*} &8.8 & 0.3 \text{ mM ATP} \\
&7.3 \\
&5.8 \end{align*}
\]
A  

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 0.03 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]

\[ \text{pH}_i 7.3 \quad 1 \text{ mM ATP} \]

\[ \text{pH}_i 6.3 \]
**A** Wild-type CFTR

- pH$_i$ 7.3
- pH$_i$ 6.3

ΔRS660A-CFTR

- pH$_i$ 7.3
- pH$_i$ 6.3

- 0.5 pA
- 100 ms

**B**

![Graph showing τ$_o$ (ms) for WT and ΔRS660A](image)

- WT
- ΔRS660A

**C**

![Graph showing τ$_{cf}$ (ms) for WT and ΔRS660A](image)

- WT
- ΔRS660A
A) Wild-type CFTR, pH$_i$ 7.3 vs. pH$_i$ 6.3

- K1250M-CFTR
- K464A-CFTR
- D572N-CFTR

B) Bar chart showing $	au_0$ (ms) for WT, K1250M, K464A, and D572N CFTR at pH$_i$ 7.3 and pH$_i$ 6.3.

- WT: 30 ms (6 measurements)
- K1250M: 30 ms (5 measurements)
- K464A: 30 ms (6 measurements)
- D572N: 40 ms (6 measurements)

C) Bar chart showing $	au_{cf}$ (ms) for WT, K1250M, K464A, and D572N CFTR at pH$_i$ 7.3 and pH$_i$ 6.3.

- WT: 4 ms (5 measurements)
- K1250M: 4 ms (5 measurements)
- K464A: 4 ms (5 measurements)
- D572N: 4 ms (5 measurements)
A pH 7.3

G551D-CFTR

pH 6.3

pH 7.3

G1349D-CFTR

pH 6.3

ΔF508-CFTR

pH 7.3

pH 6.3

0.5 pA 1 s

MBD (ms)

WT G551D G1349D ΔF508

0

200

400

20

40

60

80

100

MBD / τo

WT G551D G1349D ΔF508

0

2

4

6

8

10

WT G551D G1349D ΔF508