ATP Alters Current Fluctuations of Cystic Fibrosis Transmembrane Conductance Regulator: Evidence for a Three-state Activation Mechanism

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ABSTRACT   The cystic fibrosis gene product cystic fibrosis transmembrane conductance regulator (CFTR) is a low conductance, cAMP-regulated Cl^- channel. Removal of cytosolic ATP causes a cessation of cAMP-dependent kinase–phosphorylated CFTR channel activity that resumes upon ATP addition. (Anderson, M. P., H. A. Berger, D. R. Rich, R. J. Gregory, A. E. Smith, and M. J. Welsh. 1991. *Cell.* 67:775–784). The aim of this study was to quantify possible effects of ATP on CFTR gating. We analyzed multichannel records since only 1 of 64 patches contained a single channel. ATP increased the channel open probability (P_o) as a simple Michaelis-Menten function of concentration; the effect was half maximal at 24 μM, reached a maximum of 0.44, and had a Hill coefficient of 1.13. Since the maximum P_o was not 1, the simplest description of the effect of ATP on CFTR gating is the noncooperative three-state mechanism of del Castillo and Katz (1957. *Proceedings of the Royal Society of London.* B. 146:369–381). We analyzed current fluctuations to quantify possible changes in CFTR gating. The power density spectra appeared to contain a single Lorentzian in the range of 0.096–31 Hz. Analysis of the corner frequency (f_c) of this Lorentzian revealed that ATP increased $2\pi f_c$ as a Michaelis-Menten function with a Hill coefficient of 1.08, and it provided estimates of the ATP dissociation constant (44 μM), τ_{open} (154 ms), and the ATP-sensitive τ_{close} [(185 ms) (44 μM/[ATP] + 1)]. These results suggest that the binding reaction is rapid compared to the opening and closing rates. Assuming that there is a single set of closed-to-open transitions, it is possible to verify the outcome of fluctuation analysis by comparing fluctuation-derived estimates of P_o with measures of P_o from current records. The two values were nearly identical. Thus, noise analysis provides a quantitative description of the effect of ATP on CFTR opening. The noncooperative three-state model should serve as a basis to understand possible alterations in CFTR gating resulting from regulators or point mutations.

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disease. The epithelial cells lining the airways, pancreatic ducts, intestines, and sweat ducts of CF patients lack a cAMP-activated apical membrane Cl^- conductance (Quinton, 1990). As a result, active electrogenic Cl^- secretion is impaired in the airways, pancreas, and intestines of CF patients, and conductive Cl^- reabsorption is defective in CF sweat ducts. The gene responsible for CF has been identified, cloned, and sequenced (Riordan, Rommens, Kerem, Alon, Rozmahel, Grzelczak, Zielenski, Lok, Plavsic, Chou, Drumm, Iannuzzi, Collins, and Tsui 1989). Three types of evidence indicate that the CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), functions as the low-conductance, cAMP-activated Cl^- channels that have been identified in the apical membranes of Cl^--transporting epithelial cells (Gray, Greenwell, and Argent 1988; Tabcharani, Low, Elie, and Hanrahan 1990). First, transfection of CFTR cDNA into CF cells (Rich, Anderson, Gregory, Cheng, Paul, Jefferson, McCann, Klinger, Smith, and Welsh 1990; Drumm, Pope, Cliff, Rommens, Marvin, Tsui, Collins, Frizzell, and Wilson 1990; Cliff, Schoumacher, and Frizzell, 1992) or cells not normally expressing CFTR (Berger, Anderson, Gregory, Thompson, Howard, Maurer, Smith, and Welsh 1991; Tabcharani, Chang, Riordan, and Hanrahan, 1991) resulted in the appearance of low-conductance, cAMP-regulated Cl^- channels. Second, the introduction of mutations into CFTR altered the halide selectivity (Anderson, Gregory, Thompson, Souza, Paul, Mulligan, Smith, and Welsh, 1991a). Third, reconstitution of purified CFTR protein into planar lipid bilayers yielded a channel with properties similar to the low-conductance Cl^- channel (Bear, Li, Kartner, Bridges, Jensen, Ramjeesingh, and Riordan, 1992). Thus, there is considerable evidence that the CF gene product CFTR functions as the low-conductance, regulated Cl^- channel that is defective in CF.

CFTR channel activity can be stimulated in cell-attached patches by membrane-permeant cAMP analogues and by increasing cellular cAMP (Gray et al., 1988; Tabcharani et al., 1991; Cliff et al., 1992). The regulation of CFTR has been further defined using excised inside-out membrane patches. Tabcharani et al. (1991) showed that CFTR channels are activated by cAMP-dependent kinase (PKA) and inactivated by protein phosphatase, and they concluded that PKA phosphorylation is required for opening. Subsequently, Anderson, Berger, Rich, Gregory, Smith, and Welsh (1991b) showed that the opening of CFTR involves at least two steps. First, CFTR must be activated by PKA plus ATP, which is consistent with the observations of Tabcharani et al. (1991). Second, the continued presence of ATP is required to maintain CFTR opening. Removal of ATP caused a rapid cessation of PKA-phosphorylated channel activity that resumed after the addition of ATP. These results provide suggestive evidence in support of the hypothesis that ATP can regulate CFTR gating. However, more direct evidence is needed to advance this notion.

There is also some evidence to suggest that ATP binds to CFTR. CFTR is predicted to possess two nucleotide-binding folds (NBF's) based on its genetic sequence. ATP and nucleotide derivatives bind to synthetic peptides and purified recombinant protein of the first NBF (Thomas, Shenbagamurthi, Ysern, and Pedersen, 1991;

Hartman, Huang, Rado, Peng, Jilling, Muccio, and Sorscher, 1992). Furthermore, based on homology to other proteins, CFTR is a member of the ATP-binding cassette (ABC) family of transporter proteins (Riordan et al., 1989; Higgins, Hyde, Mimmack, Gileadi, Gill, and Gallagher, 1990). Other members of the ABC family of proteins bind ATP to transport solutes across the plasma membrane (Higgins et al., 1990). Finally, the nucleotide binding folds of CFTR contain many CF-causing mutations, suggesting that nucleotide binding is important for normal function (Tsui and Buchwald, 1991). Despite this evidence that CFTR may possess two binding sites, the relationship between cytosolic ATP and CFTR gating remains unclear.

The aim of this study was to obtain a quantitative description of the effect of ATP on CFTR gating. We used inside-out membrane patches from CFTR-transfected mouse L cells (Yang, Devor, Engelhardt, Ernst, Strong, Collins, Cohn, Frizzell, and Wilson, 1993) which contained multiple channels. CFTR channels were PKA activated before patch excision. To gain some insight regarding the possible relationship between ATP concentration and CFTR gating, we measured the open probability (P_o) and then analyzed current fluctuations. Lindemann and Van Driessche (1977) showed that analysis of blocker-induced changes in current fluctuations can provide estimates of the opening and closing rates for open-channel blockade. We reasoned that a similar approach may enable study of possible ATP-mediated changes in CFTR opening or closing. We found that measurements from current records and fluctuation analysis provide convergent descriptions of the effect of ATP on CFTR, and they are consistent with a noncooperative three-state activation mechanism. We propose that this simple three-state mechanism is the central element in a more elaborate model for CFTR gating that accounts for the possibility of additional open-shut transitions (Tabcharani et al., 1991; Haws, Krouse, Xia, Gruenert, and Wine, 1992; McCarty, McDonough, Cohen, Riordan, Davidson, and Lester, 1993). Portions of this study have appeared in abstract form (Venglarik, Schultz, Frizzell, and Bridges 1992, 1993).

MATERIALS AND METHODS

Cell Culture and Electrophysiology

CFTR-transfected mouse L cells (Yang et al., 1993) were kindly provided by Jim Wilson of the University of Michigan (Ann Arbor, MI). Cells were passaged and grown on collagen-coated plastic coverslips in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 2–6 d. Coverslips were then transferred to a heated chamber (37°C) located on the stage of an inverted microscope (Nikon; Southern Micro Instruments, Atlanta, GA). Excised inside-out membrane patches were obtained by standard techniques (Hamill, Neher, Sackmann, and Sigworth, 1982; Cliff et al., 1992; Halm and Frizzell, 1992). The pipettes were fabricated from glass (8161; Garner Glass Co., Claremont, CA) using a two-stage puller (model PP-83; Narishige USA Inc., Greenvale, NY), and they had a resistance of 2–4 MΩ in the bath solution. Current measurements were obtained with a patch-clamp amplifier that used a 50-GΩ feedback resistor (List EPC7; Medical Systems Corp., Greenvale, NY). Currents were monitored with a digital storage oscilloscope (Nicolet 310, Madison, WI) and were stored in digital form on VHS videotape using a modified audio PCM recorder (2000T; Vetter, Rebersburg, PA) for later analysis. Electrical potentials are reported with respect to the pipette interior and positive

current flows "outward" from the bath into the pipette. CFTR channels were identified based on their activation by ATP and by their amplitude with an applied potential of −60 mV in symmetric 150 mM Cl⁻ (−0.78 ± 0.05 pA).[1]

Data Acquisition and Analysis

Current records were played back in analogue form, low-pass filtered with an 8-pole Bessel filter (902LPF; Frequency Devices, Haverhill, MA) using a cut-off frequency of 200 Hz, and they were acquired at 400 Hz with a TL-1 DMA interface, pClamp software (Version 5.5.1; Axon Instruments, Foster City, CA), and an IBM-compatible computer. pClamp files were analyzed using BioPatch software (Version 3.05; Molecular Kinetics Inc., Pullman, WA). 85–480 s of channel activity at a single concentration of ATP was used to determine channel open probability (P_o) and to construct the power density spectrum (PDS).

P_o was calculated as $I/(Ni)$ using the single-channel current amplitude (i) from the amplitude histogram, the average current of the multichannel record (I), and the number of active channels in the patch (N). We determined N based on the maximum number of simultaneously open channels observed in the presence of ≥ 0.3 mM ATP (Horn, 1991). Preliminary estimates revealed that 0.3 mM was the minimal ATP concentration needed to optimize P_o for determination of the zero current level and N (Horn, 1991; Marunaka and Eaton, 1991). Since it was possible to underestimate N (Horn, 1991), we derived the statistical confidence of these estimates. Based on stochastic theory and a two-state model of gating, the probability of observing simultaneous openings or simultaneous closures of all N channels during a given time interval are expected to conform to a Poisson distribution (Colquhoun and Hawkes, 1982, 1983; Hoel, Port, and Stone, 1971; Lipschultz, 1965). Based on this distribution function, a record must be of sufficient length to ensure an average of three all-open or all-closed events to guarantee that at least one of these events is observed ($P > 0.95$). Marunaka and Eaton (1991) noted that the average time needed to observe a single event in a multichannel record is the mean duration of that event derived from rate theory (Colquhoun and Hawkes, 1981, 1982, 1983) divided by the probability of observing the event that can be obtained from the binomial distribution (Colquhoun and Hawkes, 1983; Horn, 1991). Thus, the time needed to observe at least a single all-open event is: $(3\tau_{open}/N)/(P_o)^N$, and the time needed to observe at least one all-shut event (zero current level) is $(3\tau_{close}/N)/(1 - P_o)^N$ for $P > 0.95$. We can calculate that 62 s is needed to determine if eight channels are present in a patch, and 132 s is needed to exclude the possibility that there are nine channels ($\tau_{open} = 0.154$ s, $\tau_{close} = 0.214$ s, $P_o = 0.418$, and $P > 0.95$). Most records (80%) were of adequate length to insure a $P > 0.95$.

PDS were constructed and analyzed to quantify possible ATP-induced changes in CFTR gating (for review see DeFelice, 1981; Van Driessche and Van Deynse, 1990; Eaton and Marunaka, 1990). Records were divided into nonoverlapping 20.48-s segments containing 8,192 data points, and the PDS was calculated for each block using BioPatch software. We averaged 4–23 spectra that represented 84–450 s of current recording to reduce the variance within the PDS. These data were fit by a single Lorentzian type function:

$$S = S_0/(1 + (f/f_c)^2) \tag{1}$$

S_0 is the zero frequency asymptote, and f_c is the corner frequency, where S_0 has been decreased by half. The corner frequency is related to a set of opening (r_+) and closing (r_-) rates:

$$2\pi f_c = r_+ + r_- \tag{2}$$

It is important to distinguish between the opening or closing rates (i.e., r_+ and r_-), which may not have a simple physical significance, and the underlying chemical rate constants (Colquhoun

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[1] Arithmetic mean ± SD of the single-channel current estimates from 64 experiments.

and Hawkes, 1982, 1983; Van Drissche and Van Deynse, 1990). Lindemann and Van Driessche (1977) used spectral analysis to obtain the kinetic constants for open-channel blockade by plotting $2\pi f_c$ as a function of blocker concentration and fitting the result to the expected outcome derived from rate theory. We reasoned that a similar approach would enable quantification of possible ATP-mediated changes in CFTR gating. Since a kinetic model is required to derive the expected relationship between ATP concentration and $2\pi f_c$, and since the selection of a model depends on the outcome of the P_o measurements, further detail regarding this analysis is presented in Results.

The ATP-induced changes in P_o and $2\pi f_c$ were quantified by plotting the data as a function of ATP concentration and fitting the outcome to Michaelis-Menten functions using Statgraphics software (Version 5.0; STSC Inc., Rockville, MD). Figures were plotted for presentation using Sigmaplot software (Version 4.2; Jandel Scientific, San Rafael, CA).

Materials

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except for NaF, which was obtained from Fisher Scientific (Pittsburgh, PA). The pipette contained (in mM): 160 N-methyl-D-glucamine-HCl (NMGD-Cl), 1 $CaCl_2$, 2 $MgCl_2$, 10 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (BTP). The bathing solution contained (in mM): 150 NaCl, 5 KCl, 2 $MgCl_2$, 10 NaF, 0.5 ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.2 $CaCl_2$, and 10 BTP. All solutions were titrated to pH 7.4. After patch excision, the 0.75-ml bath was continually perfused at a rate of 1.8 ml/min. ATP was added to perfusion solutions as a small volume of a concentrated stock solution (200 mM) containing 200 mM $MgCl_2$ and 200 mM BTP. Since the bathing solution always contained a 2-mM excess of free Mg^{+2}, 96% of the ATP is expected to be complexed with Mg^{2+} over the range of concentrations used in this study (Chang, Hsieh, and Dawson, 1988). The addition of ATP is also not expected to alter the free Ca^{+2} concentration, which was calculated to be 57 nM (Chang et al., 1988). The purity of the ATP was tested by nuclear magnetic resonance and found to be $>98\%$.

RESULTS

ATP Increases P_o as a Michaelis-Menten Type Function

To gain some general insight into the possible relationship between ATP and CFTR channel gating, we quantified the relationship between ATP concentration and P_o. CFTR channels were PKA phosphorylated by raising cellular cAMP before excision. Inside-out patches were then obtained using standard techniques and exposed to varying concentrations of bath ATP (see Materials and Methods). Upon excision, the bath potential was held at -60 mV. The resulting inward current provided a selective measure of outward Cl^- flow, since the pipette solution contained the impermeant cation $NMDG^+$. In a majority of excised patches, the number of active channels appeared to be stable for periods of 7–15 min in the absence of PKA. We obtained further evidence for a very slow rate of dephosphorylation by observing a constant number of active channels in experiments performed with sufficient ATP (i.e., 0.3–1 mM) to provide a reliable estimate of N ($P > 0.95$) during the entire 4–64 min lifespan of 12 excised patches (data not shown).

Initially, 5–10 μM forskolin was used to raise cellular cAMP and presumably activate endogenous PKA. However, we discovered that addition of forskolin was unnecessary, since CFTR was spontaneously active in cell-attached patches when the cells were bathed by a solution containing 10 mM NaF. We did not observe any

cell-attached Cl^- channel activity in CFTR-transfected mouse L cells in the absence of bath NaF or forskolin ($n = 16$). The addition of 10 mM NaF-activated channels in cell-attached patches within 5–10 min ($n = 9$) and the subsequent addition of forskolin did not cause any apparent increase in channel number ($n = 5$). This result indicates that NaF and forskolin act on the same channel population, and it is consistent with previous observations that F^- can activate adenylate cyclase (Rall and Sutherland, 1958; Sternweis and Gilman, 1982; Domínguez, Barros, and Lazo, 1985).
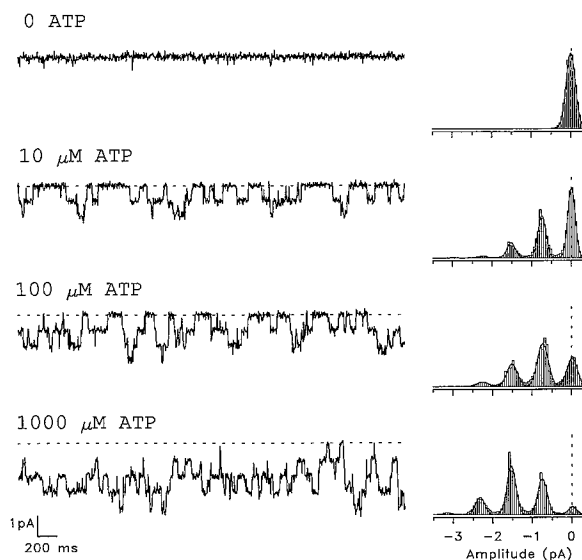

FIGURE 1. Comparison of CFTR activity in the presence of 0, 10, 100, and 1,000 μM ATP. The records and histograms in the presence of ATP were obtained from a single excised patch containing four channels with an amplitude of 0.76 ± 0.11 pA (mean ± SD from fit). The record showing no apparent channel activity in the absence of ATP was obtained from another patch that contained seven active channels, and it is representative of 16 experiments performed with 0 ATP. This result combined with the observation of a single ATP-sensitive channel in 27 excised membrane patches from the parental mouse L cell line provides additional evidence that the ATP-sensitive Cl^- channel activity was caused by the transfected human CFTR and was not the result of endogenous channels. All records were low pass filtered with a cut-off of 200 Hz using an 8-pole Bessel filter and acquired at 400 Hz. Each amplitude histograms was constructed from 123 s of data and fit to Gaussian functions using a nonlinear least-squares routine (BioPatch) as shown by the solid lines. The histograms in the presence of ATP contain identical areas. However, because of scaling limitations, the area of the histogram in the absence of ATP was reduced by one third. This figure was constructed using SigmaPlot software and BioPatch files of current records, amplitude histograms, and fits. The pipette contained (in mM): 160 NMGD Cl, 1 $CaCl_2$, 2 $MgCl_2$, and 10 BTP-HCl (pH 7.4). The 0.75-ml bath was continually perfused at a rate of 1.8 ml/min with a solution containing (in mM): 150 NaCl, 5 KCl, 2 $MgCl_2$, 10 NaF, 0.5 EGTA, 0.2 $CaCl_2$, 10 BTP-HCl (pH 7.4), and ATP (as indicated). The holding potential was −60 mV.

However, we cannot exclude the possibility of another mechanism of activation. We also tested for possible effects of 1 mM extracellular ATP and observed no activity, although CFTR channels were present and became active after addition of 5 μM forskolin ($n = 7$).

Fig. 1 shows representative current records and amplitude histograms of CFTR activity recorded from excised membrane patches in the presence of 0, 10, 100, and 1,000 μM ATP. The single-channel current amplitude was −0.76 ± 0.11 pA, which is

the result expected for CFTR (Tabcharani et al., 1991; Anderson et al., 1991*b;* Cliff et al., 1992). The current records and amplitude histograms show that anion channel activity was increased by raising the bath (cytosolic) ATP concentration, which is also the result expected for CFTR (Anderson et al., 1991*b*, Anderson and Welsh, 1992*a*; Nagel, Hwang, Nastiuk, Nairn, and Gadsby, 1992). Having shown that ATP increased
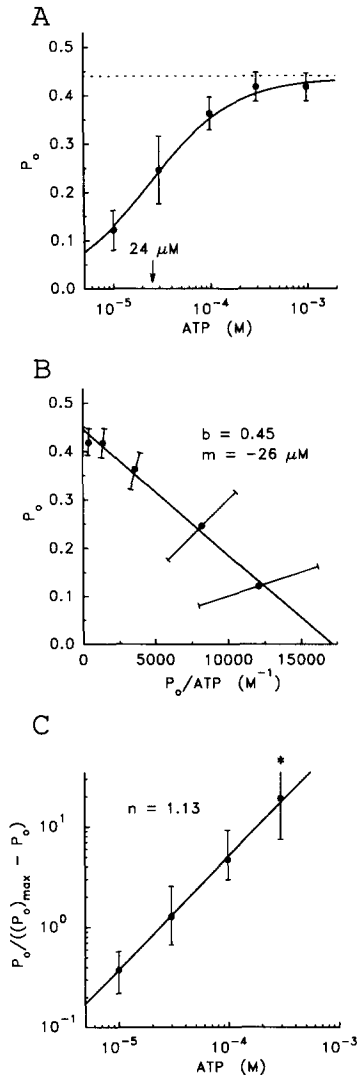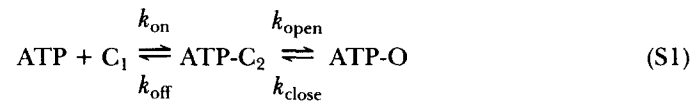

FIGURE 2. Log dose–response relation of the effect of ATP on P_o (*A*) and the Eadie-Hofstee (*B*) and Hill (*C*) plots of these data. This figure summarizes the results from 64 experiments that were performed under the conditions described in Fig. 1. Each point represents the arithmetic mean of ≥ 10 different experiments (refer to Table I), and error bars indicate $\pm$ SEM. *A* shows the log dose–response relation of P_o calculated as $I/(Ni)$. The confidence level for N was > 0.95 for 64 estimates and $0.90 < P < 0.95$ for the remaining 16. The solid line illustrates the best fit of the entire data set to a Michaelis-Menten type equation ($P_o = (P_o)_{max} \cdot [ATP]/ (K_m + [ATP])$) using nonlinear curve fitting software (Statgraphics, STSC Inc., Rockville, MD). The fit provided estimates of the Michaelis constant ($K_m = 24 \pm 8.4$ μM) and the maximum open probability (($P_o)_{max} = 0.44 \pm 0.03$). *B* shows the Eadie-Hofstee plot of the mean values of these data ($\pm$SEM). The solid line was obtained by simple linear regression. The slope ($m = -26$ μM) and y intercept ($b = 0.45$) are indicated in the plot and provide estimates of $-K_m$ and $(P_o)_{max}$, respectively. *C* shows the Hill plot of the effect of ATP concentration on P_o. The solid line was also obtained by performing simple linear regression of the mean values, and it provides an estimate of the Hill coefficient ($n = 1.13$). The asterisk indicates that the mean + SEM was undefined.

the activity of CFTR channels in excised membrane patches, we analyzed results from additional experiments to quantify the effect. Fig. 2 *A* shows the log dose–response relation of P_o for 64 excised membrane patches as a function of ATP concentration. The mean, SEM, and number of observations at each concentration (n) are summarized in Table I. The sigmoid appearance of the P_o on a log dose–response plot is

consistent with a Michaelis-Menten function and, therefore, we fit the data set to this function using nonlinear regression (*solid line*). The maximum open probability [$(P_o)_{max}$] was 0.44 ± 0.03, and the Michaelis constant (K_m), which is the ATP concentration required for a 50% increase in P_o, was 24 ± 8 μM. The fit was in good agreement with these data and thus provides suggestive evidence for a simple three-state gating mechanism that is discussed below. Fig. 2 *B* shows the Eadie-Hofstee plot of the relationship between ATP concentration and P_o. The ability to describe these data with a straight line provides further evidence that the effect of ATP on P_o is consistent with a simple Michaelis-Menten function. In addition, the linear plot provided estimates of $(P_o)_{max}$ (0.45) and K_m (26 μM) that are similar to the values obtained from the nonlinear curve fit.[2] Finally, the Eadie-Hofstee plot allows our data to be compared with the results contained in an earlier report (Anderson and Welsh, 1992*a*, 1992*b*). The two plots are markedly dissimilar, suggesting a difference in ATP binding. This possibility is considered in the Discussion.

Since the effect of ATP on P_o is well described by a Michaelis-Menten function, and since the maximum P_o observed in the presence of saturating ATP concentrations was < 1, the simplest mechanism for CFTR gating is the del Castillo and Katz (noncooperative) mechanism that is similar to formulas used in enzyme kinetics (del Castillo and Katz, 1957; Colquhoun and Hawks, 1981, 1982):

$$\text{ATP} + \text{C}_1 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{ATP-C}_2 \underset{k_{close}}{\overset{k_{open}}{\rightleftharpoons}} \text{ATP-O} \tag{S1}$$

In this mechanism, ATP binds to a closed CFTR-receptor (C_1), leading to the formation of an intermediate ATP-occupied closed state (ATP-C_2). Subsequently, the intermediate closed state (ATP-C_2) can undergo transitions to and from the open state (ATP-O). Inclusion of the intermediate ATP-occupied closed state is necessitated by the fact that the maximum P_o was < 1, which excludes the possibility of a simple two state mechanism. The rate constants, k_{on}, k_{off}, k_{open}, k_{close}, are defined by scheme S1. It is important to note that the aim of this study was to quantify the effect of ATP on CFTR gating and that the three-state model provided the simplest mechanistic interpretation of our data (see below). The possibility and implications of additional states are considered in Discussion.

In the three-state model, P_o is related to ATP concentration by an equation of the Michaelis-Menten form:

$$P_o = \frac{(P_o)_{max}[\text{ATP}]}{R \cdot \dfrac{k_{off}}{k_{on}} + [\text{ATP}]} \tag{3}$$

$(P_o)_{max}$ is the maximum open probability and the Michaelis constant (K_m) is the product of the apparent dissociation constant for ATP (k_{off}/k_{on}) and a constant (R),

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[2] Although the Eadie-Hofstee plot is considered to be the most accurate linear plot, the variation shown by the error bars in Fig. 2 *B* is inconsistent with least squares solution used by simple linear regression (Wilkinson, 1961; Munson and Rodbard, 1983). Therefore, results from the nonlinear curve fit are expected to be more reliable and will be used for comparison.

which is determined by the opening and closing rate coefficients. We derive this function and discuss the relationship between k_{off}/k_{on} and the K_m in the Appendix.

As an additional test of the simple three-state gating mechanism, we constructed a Hill plot of the ATP-induced change in P_o, which is shown in Fig. 2 C. The line shows the best fit of the transformed data to a straight line using linear regression. The Hill coefficient (1.13) was not different from 1, indicating that ATP binding is noncooperative over the range of ATP concentrations tested. The ability to describe these data with simple Michaelis-Menten functions (Figs. 2, *A* and *B*) and the lack of cooperativity (Fig. 2 C) provide preliminary support for the simple three state gating mechanism shown in S1.

The Power Density Spectra Contained a Single ATP-sensitive Lorentzian Component

Nearly all excised patches contained multiple channels (i.e., 63 of 64). Therefore, we decided to quantify possible ATP-induced changes in gating by analyzing the current fluctuations. However, to proceed with this analysis, it was necessary to satisfy two criteria. First, we needed to show that the PDS contained a well-defined Lorentzian component, since it is possible that CFTR channels show heterogeneous gating or that the transitions do not occur within a detectable range. Second, we needed to obtain evidence that the corner frequency (f_c) of the Lorentzian component varied as a function of ATP concentration. Since the corner frequency of the Lorentzian component is related to the sum of the apparent opening and closing rates (Eq. 2), the absence of an effect of ATP on f_c would preclude further analysis. Fig. 3 shows representative PDS in the presence of 0, 10, 100, and 1,000 μM bath ATP. These PDS were obtained from the same experiments used to construct the current records and amplitude histograms illustrated in Fig. 1. In the absence of ATP, there was no obvious Lorentzian component, and the PDS is consistent with electronic and seal noise. After the addition of ATP, the noise increased and the PDS appeared to contain a single Lorentzian component in the frequency range of 0.096–31 Hz. The PDS was dominated by instrument and/or seal noise at higher frequencies (>31 Hz; Schultz, B. D., C. J. Venglarik, R. A. Frizzell, and R. J. Bridges, unpublished observations), and the range of the nonlinear fit (0.096–31 Hz) was selected to exclude this component. The solid lines show the best fit of the data in each spectrum to a single Lorentzian function (i.e., Eq. 1). The excellent agreement between the data and the fits in Fig 3 suggests that CFTR undergoes a single set of closed-to-open transitions in the frequency range of 0.096–31 Hz.

The appearance of a single Lorentzian component is somewhat surprising, since a three-state gating mechanism should produce two sets of closed-to-open transitions and two Lorentzians (Colquhoun and Hawkes, 1981, 1982). However, the presence of a single set of transitions is consistent with the three-state gating mechanism provided that either (a) the on and off rates for ATP are not rate limiting (Anderson and Stevens, 1973), or (b) the expected brief closed periods within bursts are too brief to be resolved (Colquhoun and Hawkes 1981, 1982; Colquhoun and Sakmann, 1985). We will consider these two hypotheses in following sections. It is also possible that the appearance of a single set of closed-to-open transitions is caused by relatively slow on and off rates for ATP (i.e., time constant > 125 s). However, the relatively rapid onset of the effects of ATP (Anderson et al., 1991b; Haws et al., 1992; Nagel et

al., 1992) and the absence of low-frequency or 1/f noise that was not accounted for by the single Lorentzian fits (Fig. 3) argue against this hypothesis.

A comparison of the Lorentzian components in the presence of 10, 100, and 1,000 μM ATP shown in Fig. 3 also revealed that the corner frequency increased as a saturating function of ATP. This result excludes the hypothesis that the on rate for ATP is relatively slow and suggests that one of the transition rates is increased as a saturating function of ATP concentration. Based on the three-state mechanism, the opening rate is expected to increase as a saturating function of ATP concentration, and the apparent closing rate is predicted to be independent of ATP (see below).
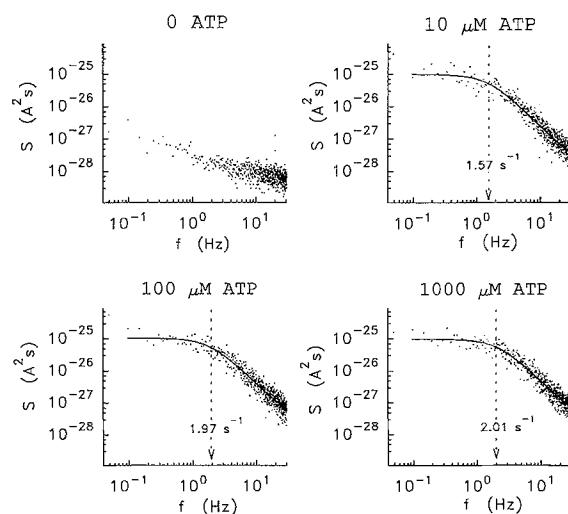

FIGURE 3. Representative power density spectra in the presence of 0, 10, 100, and 1,000 μM ATP. These PDS were calculated from the same records used to construct the amplitude histograms shown in Fig. 1. Current records were low pass filtered with a cut-off of 200 Hz using an 8-pole Bessel filter and acquired at 400 Hz. The records were divided into 8,192-point segments (20.48 s), and the PDS was calculated for each segment using BioPatch software. Each plot illustrates the average of six uncorrected spectra, and they are representative of the outcome of 80 experiments in the presence of ATP and 16 experiments with 0 ATP. The solid lines illustrate the best fit of the data to a single Lorentzian type function (Eq. 1) obtained using a nonlinear least squares fitting routine (BioPatch). The lowest data point (0.048 Hz) was excluded from the fit because of our limited confidence in that estimate. The second data point (0.096 Hz) corresponds to the fundamental frequency of 12 averaged spectra and is more reliable. The arrows indicate the corner frequency (f_c) from the Lorentzian fit. The outcome of the fit was not dependent on the square, Hanning, Parze, or Welch window functions (not shown) and, therefore, all data was analyzed using a square window. Experimental conditions are as given in Fig. 1.

Furthermore, the 28% increase in f_c from 1.57 to 2.01 can account for a >50% increase in P_o for a channel with a $(P_o)_{max} < 0.5$. Thus, in addition to satisfying both criteria needed to proceed with current fluctuation analysis, Fig. 3 contains suggestive evidence in support of the three-state gating model. In the following section, we will rigorously test this hypothesis by deriving the equation for the opening and closing rates based on the three-state gating mechanism, and by comparing the plot of $2\pi f_c$ as a function of ATP concentration with the predicted outcome.

The Effect of ATP on $2\pi f_c$ Supports the Three-state Gating Mechanism

To quantify possible ATP-induced changes in CFTR gating using noise analysis, it was necessary to derive the expected relation between ATP concentration and $2\pi f_c$ based on the three-state model. This was achieved by combining the predicted opening and closing rates for the three-state gating mechanism with Eq. 2. Colquhoun and Hawks (1981, 1982, 1983) derive the opening and closing rates for the three-state model (S1). The ATP-sensitive opening rate (r_+) can be obtained from the product of the probability that a closed channel is in the intermediate closed state $[k_{on}[ATP]/(k_{off} + k_{on} [ATP])]$ and the opening rate constant (k_{open}). Rearranging the resulting relation yields:

$$r_+ = \frac{k_{open}[ATP]}{\dfrac{k_{off}}{k_{on}} + [ATP]} \tag{4}$$

Thus, the opening rate (r_+) is predicted to increase as a Michaelis-Menten type saturating function of ATP concentration with a maximum value of k_{open} and a Michaelis constant corresponding to the apparent dissociation constant for ATP (k_{off}/k_{on}). The derivation of the apparent closing rate (r_-) depends on the explanation for the presence of a single set of closed-to-open transitions. If the on and off rates for ATP are relatively fast, then r_- is approximated by the product of the probability that an open channel is in the single open state (~ 1) and the closing rate constant (k_{close}). Thus:

$$r_- = k_{close} \tag{5a}$$

Alternatively, if brief closures are excluded from analysis then r_- is inversely related to the mean burst length (T_{burst}):

$$r_- = T_{burst}^{-1} \tag{5b}$$

Eq. 5 shows that the apparent closing rate is a constant despite the ambiguity regarding its mechanistic interpretation. However, because of this ambiguity, the apparent closing rate is best represented by its most general form (r_-). Combining the opening rate (Eq. 4) and Eq. 2 yields:

$$2\pi f_c = \frac{k_{open} \cdot [ATP]}{\dfrac{k_{off}}{k_{on}} + [ATP]} + r_- \tag{6}$$

Thus, the value of $2\pi f_c$ is expected to vary as a Michaelis-Menten type function of ATP concentration (opening rate) plus a constant (apparent closing rate). A favorable comparison between the predicted effect of ATP concentration on $2\pi f_c$ as given by Eq. 6, and the observed effect will provide strong support for the hypothesis of a three-state gating mechanism and should yield estimates of the transition rates and kinetic constants.

Fig. 4 *A* shows a plot of the mean and SEM of $2\pi f_c$ as a log function of ATP concentration for the series of experiments used to construct Fig. 2. The solid line
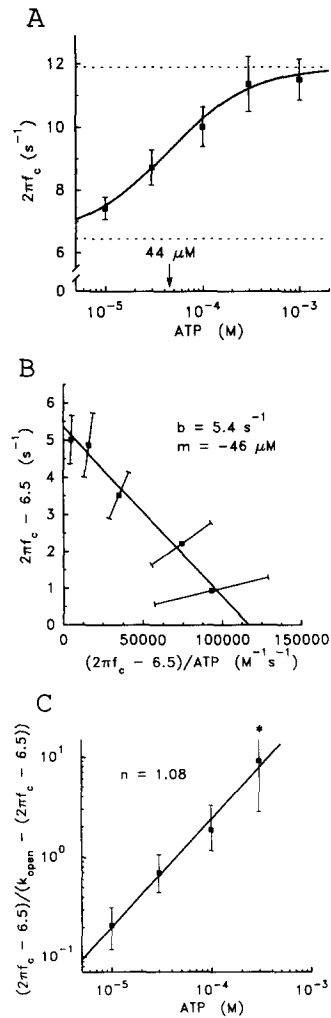
FIGURE 4. Log dose–response relation of the effect of ATP on $2\pi f_c$ (A) and the Eadie-Hofstee (B) and Hill (C) plots of the opening rate calculated as: $2\pi f_c$–6.5 s^{-1}. These data were obtained from fluctuation analysis of the 64 experiments used to construct Fig. 2. The PDS was calculated for each record and fit to a single Lorentzian type function to obtain an estimate of f_c (refer to Fig. 3). Each point represents the arithmetic mean of ≥ 10 different experiments and error bars represent the SEM (see Table I). A shows the log dose–response relation of $2\pi f_c$ as a function of ATP. The solid line illustrates the best fit of these data to a Michaelis-Menten type function plus a constant (i.e., Eq. 6) using a nonlinear curve fitting routine (Statgraphics). Based on Eq. 6, the fit provides estimates of r_- (6.5 ± 0.5 s^{-1}), k_{off}/k_{on} (44 ± 14 μM), and k_{open} (5.4 ± 0.4 s^{-1}). The dotted lines illustrate the expected range of $2\pi f_c$ and correspond to r_- (*bottom line*) and $r_- + k_{open}$ (*top line*). Based on these values, f_c is expected to range from 1.0 ± 0.1 to 1.9 ± 0.1 Hz. Part B shows the Eadie-Hofstee plot of the ATP-sensitive opening rate ($2\pi f_c$ – 6.5 s^{-1}). The solid line was obtained by simple linear regression. The slope ($m = -46$ μM) and y intercept ($b = 5.4$ s^{-1}) are indicated in the plot and provide estimates of $-k_{off}/k_{on}$ and k_{open}, respectively. The r value of the fit was 0.991. C shows the Hill plot of the effect of ATP concentration on the opening rate. The solid line was also obtained by simple linear regression. The fit provided an estimate of the Hill coefficient ($n = 1.08$) and had an r value of 0.994. The asterisk indicates that the mean + SEM was undefined. Refer to Figs. 1 and 2 for additional detail regarding experimental conditions.

illustrates the best fit of the mean values to Eq. 6. The excellent agreement between the observed values of $2\pi f_c$ and the predicted function supports the hypothesis of a three-state gating mechanism. In addition, the fit provides estimates of the kinetic constants: $r_- = 6.5 \pm 0.5$ s^{-1}, $k_{open} = 5.4 \pm 0.4$ s^{-1}; and $k_{off}/k_{on} = 44 \pm 14$ μM. Since the open and closed time constants are inversely related to the transition rates, the estimate of r_- can be used to calculate an ATP-insensitive apparent open time

(τ_{open} = 154 ± 12 ms). Similarly, Eq. 5 can be inverted to derive an estimate of the ATP-sensitive closed times [τ_{close} = (185 ms)(44 μM/[ATP] + 1)].[3] At maximal ATP concentrations, the probability that CFTR is not bound to ATP approaches zero, the three-state model is approximated by a two-state model, and the record will appear to contain a single population of closed events. The minimum closed time at saturating ATP concentrations (185 ± 18 ms) indicates that k_{open} is rate limiting compared to k_{on}. Therefore, the three-state gating mechanism is not expected to produce frequent brief gaps within bursts. We provide additional support for this notion in the following section by showing that brief closures do not make a significant contribution to P_o.

Table I summarizes the mean and SEM for $2\pi f_c$ at each ATP concentration tested. Since the closing rate is constant (6.5 s^{-1}), it is possible to calculate the opening rate using Eq. 2 and the values of $2\pi f_c$:

$$r_+ = 2\pi f_c - 6.5 \text{ s}^{-1} \tag{7}$$

TABLE I

Effect of ATP on P_o and $2\pi f_c$

ATP (μM)	P_o (I/(Ni))	$2\pi f_c$ (s^{-1})	P_o ((2πf_c-6.5)/2πf_c)	n	S
10	0.121 ± 0.041	7.43 ± 0.36	0.125 ± 0.045	10	46
30	0.246 ± 0.070	8.72 ± 0.56	0.255 ± 0.051	12	74
100	0.363 ± 0.034	10.02 ± 0.62	0.351 ± 0.038	16	76
300	0.418 ± 0.030	11.37 ± 0.87	0.428 ± 0.047	12	81
1000	0.417 ± 0.029	11.51 ± 0.65	0.435 ± 0.034	30	231

Values are means ± SEM. This table summarizes the results shown in Figs. 2, 4, and 5. The number of experiments performed at each ATP concentration (n) and the total number of 20.48 s intervals (S) used to obtain the estimates of I/Ni and $2\pi f_c$ are also presented. Refer to Figs. 1–3 for a description of the experimental conditions and for details regarding the analysis and calculations.

This equation was used to calculate the opening rate using the values of $2\pi f_c$ in Table I. Fig. 4, *B* and *C* illustrate these opening rates as Eadie-Hofstee and Hill plots, respectively. The lines show the best fit of the transformed mean values using simple linear regression. The ability to describe the Eadie-Hofstee plot with a straight line indicates that the opening rate is a Michaelis-Menten function, as expected for the three-state gating mechanism (i.e., Eq. 4). The intercept (*b*) and slope (*m*) of the line provide estimates of k_{open} (5.4 s^{-1}) and $-k_{off}/k_{on}$ (−46 μM) that are similar to the values obtained from the nonlinear curve fit. In addition, the Hill coefficient (n = 1.08) was not different from 1, indicating that ATP binding is noncooperative. The plot of the effect of ATP concentration on $2\pi f_c$, the Eadie-Hofstee plot of the opening rate, and the Hill plot of the opening rate provide excellent support for a noncooperative three-state gating mechanism.

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[3] Note that this equation for τ_{close} yields a reciprocal plot that is similar to the Lineweaver-Burk plot and may be useful for single-channel analysis.

Comparison of the Fluctuation-derived Estimates of P_o with the P_o from Current Records

In the two previous sections, we analyzed CFTR current fluctuations, showed that CFTR appears to undergo a single set of closed-to-open transitions, and obtained estimates of the kinetic constants. These results were in excellent agreement with the three-state gating mechanism predicted from the ATP-induced increase in CFTR open probability. However, current fluctuation analysis relies on a number of presumptions, and the conclusions are subject to some uncertainty. Therefore, it was necessary to conduct an additional test of the results from noise analysis. In addition, we wanted to address the hypothesis that a single set of closed-to-open transitions can account for all of the changes in P_o observed with ATP. It is possible to satisfy both of these aims by assuming that CFTR undergoes a single set of closed-to-open transitions, calculating the P_o based on the results obtained from current fluctuation
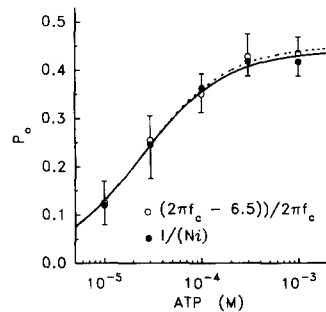

FIGURE 5. Comparison of the fluctuation-derived P_o and the P_o from current records. Assuming that CFTR undergoes a single set of closed-to-open transitions, it is possible to calculate the P_o directly from the values of $2\pi f_c$ in Fig. 4 A and Table I using the estimate of the apparent closing rate (6.5 s^{-1}) and Eq. 8. This figure depicts the log dose–response relation of the fluctuation-derived P_o [$(2\pi f_c - 6.5\ s^{-1})/2\pi f_c$] as a function of ATP (*open circles*). Each point represents the arithmetic mean of ≥ 10 experiments previously described in Figs. 2 and 4. Error bars are SEM. The dashed line illustrates the best fit of these data to a Michaelis-Menten type function (i.e., Eq. 3) using Statgraphics software (STSC Inc.). The fit provided estimates of K_m (24 ± 2.2 μM) and $(P_o)_{max}$ (0.45 ± 0.01). The P_o from current records (*solid circles*) and the fit of these data to a Michaelis-Menten type function (*solid line*) are presented for comparison.

analysis, and comparing the outcome with the P_o obtained directly from current records.

We estimated P_o from the values of $2\pi f_c$ in Table I and the apparent closing rate obtained from the fit of Fig. 4 A (6.5 s^{-1}). Assuming that CFTR undergoes a single set of transitions, it is possible to calculate P_o by dividing the opening rate (Eq. 7) by the sum of the opening and closing rates (Eq. 2):

$$P_o = (2\pi f_c - 6.5\ s^{-1})/2\pi f_c \tag{8}$$

The P_o was calculated for each concentration of ATP using Eq. 8, and the results are summarized in Table I. Fig. 5 shows a plot of these data as a log function of ATP concentration (*open circles*). The dashed line shows the best fit of the data to a Michaelis-Menten type function. The data and fit shown in Fig. 2 A are plotted for comparison. Note that the two estimates of P_o are nearly identical at every concentration tested. The Michaelis constants were identical for both data sets (24 ± 2 vs

24 ± 8 μM) and $(P_o)_{max}$ was increased only slightly (0.45 ± 0.01 vs 0.44 ± 0.03). It is possible to complete this comparison by using the estimates of the kinetic constants from current fluctuation analysis to calculate $(P_o)_{max}$ and K_m based on Eq. 12, which is contained in the Appendix. The predicted $(P_o)_{max}$ is: $1 \cdot [5.4 \ s^{-1}/(5.4 \ s^{-1} - 6.5 \ s^{-1})] = 0.45$, and the calculated K_m is $[6.5 \ s^{-1}/(5.4 \ s^{-1} + 6.5 \ s^{-1})](44 \ \mu M) = 24 \ \mu M$. These values also are nearly identical to the results obtained from the fit of the data in Fig. 2 A to a Michaelis-Menten function.

The remarkable agreement between the two measures of P_o in Fig. 5 eliminates much of the uncertainty regarding the presumptions underlying current fluctuation analysis. The values of P_o from the current records are entirely model independent, and they are accurate regardless of the number and/or frequency of open and closed states. The two measures are expected to agree only if CFTR gating is dominated by the single set of low-frequency transitions, and if the presumptions that underlie current fluctuation analysis are valid. Thus, the comparison of the P_o provides compelling evidence that CFTR channels form a homogeneous population in mouse L cells and gate according to the noncooperative three-state mechanism. Furthermore, the comparison shows that the single set of closed-to-open transitions accounts for 98% of the ATP-induced change in CFTR open probability. Although we can not exclude the possibility that CFTR can undergo additional brief or infrequent events, the change in P_o is clearly dominated by the single set of transitions under our experimental conditions.

DISCUSSION

Evidence for a Three-State Gating Mechanism

We have quantified the effect of ATP on CFTR using two different parallel means of analysis and provide converging lines of evidence for a noncooperative three-state model of CFTR gating. To our knowledge, this is the first time these two measures of channel activity (I/N_i and $2\pi f_c$) have been used in parallel to analyze the same data. The comparison between the two estimates of P_o in Fig. 5 validates the notion that both analyses are being performed properly, since it is virtually impossible to have a systematic error in I/N_i and/or $2\pi f_c$ and to maintain the near identity shown.

Our results show that the PDS of CFTR contained a single Lorentzian (Fig. 3), and that ATP concentration increased $2\pi f_c$ as a Michaelis-Menten type function (Fig. 4). These observations are consistent with a simple three-state gating mechanism predicted by the effect of ATP concentration on P_o (Fig. 2). The fit of these data to the expected function provided estimates of the apparent dissociation constant for ATP (44 ± 14 μM), the mean open time (154 ± 12 ms), and the ATP-sensitive mean closed time $[(185 \ ms)(44 \ \mu M/[ATP] + 1)]$. The minimum mean closed time (185 ± 18 ms) observed in the presence of maximal ATP concentrations indicates that k_{open} is rate limiting, and it explains the appearance of a single Lorentzian component in the PDS. Furthermore, we were able to verify the three-state model and our estimates of the kinetic constants by showing that the fluctuation-derived estimates of P_o were nearly identical to the measures of P_o taken directly from current records (Fig. 5). The agreement also indicates that the single set of closed-to-open transitions dominates CFTR P_o.

Based on these results, we can conclude that the three-state model provides an accurate description of the effect of ATP on CFTR gating. This model and our estimates of the kinetic constants should provide the means to interpret and quantify possible alterations in CFTR gating caused by regulators or point mutations. In addition, we have devised an approach to study multichannel records that combine two parallel means of analysis to quantify changes in gating. This approach should be useful in future studies of CFTR and has possible application to other types of ligand-activated channels.

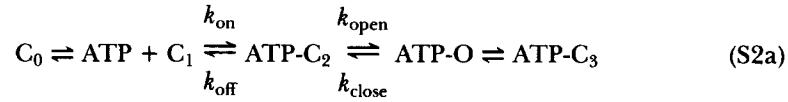### Comparison of the Fluctuation-derived Kinetic Constants

Our estimates of the kinetic constants compare favorably to several previous reports. Based on the number of transitions, the record duration, and number of active channels, Gray, Greenwell, and Argent (1988) calculated τ_{open} (135–262 ms) and τ_{close} (306–1468 ms) for CFTR in pancreatic ductal cells. We used a different approach to analyzing multichannel records (noise analysis) and obtained similar estimates ($\tau_{open} = 154 \pm 12$ ms; $\tau_{close} \geq 185 \pm 18$ ms). More recently, current fluctuation analysis of gradient-driven Cl^- secretion across monolayers of HT-29/B6 human colonic cells revealed that the CFTR PDS contained a single low frequency Lorentzian component with an f_c of 1.4 ± 0.2 Hz (Fischer, Kreusel, Illek, Machen, Hegel, and Clauss, 1992). This value is consistent with the range of f_c's from the fit of the data in Fig. 4A (1.0 ± 0.01 to 1.9 ± 0.2 Hz). There is also preliminary evidence for a low frequency f_c of 1.6 ± 0.1 Hz in cell-attached recordings from CFTR-transfected 3T3 cells (Fischer and Machen, 1993). The identification of similar Lorentzian components in an intact Cl^--secreting epithelium and in cell-attached patches from another heterologous expression system argues that the three-state gating mechanism is well conserved between these different cells and recording techniques. However, there are several reports of longer apparent open times (Dalemans, Barbry, Champigny, Jallat, Dott, Dreyer, Crystal, Pavirani, Lecocq, and Lazdunski, 1991; Haws et al., 1992; Li, Ramjeesingh, Reyes, Jensen, Chang, Rommens, and Bear, 1993) and additional study is needed to advance this notion.
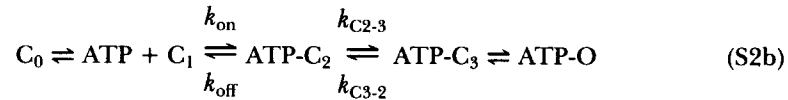
Implications of the Three-State Model

The three-state model was the simplest model that accounted for the effects of ATP on P_o, and it provided an excellent description of the effect of ATP on CFTR gating under our recording conditions. However, there are at least two lines of evidence that the three-state mechanism may be part of a more elaborate scheme. First, the use of PKA or agents that increase cellular cAMP to activate CFTR implies the existence of at least one dephosphorylated closed state (Tabcharani et al., 1991; Anderson et al., 1991b; Cliff et al., 1992; Hwang, Nagel, Nairn, and Gadsby, 1993). Second, the observation of brief closed events that are too frequent to be accounted for by the three-state model (i.e., <2 ms) suggests that CFTR can enter an additional short-lived closed state from the open state (Gray et al., 1988; Bear et al., 1992; Dalemans et al., 1991; Cliff et al., 1992; Haws et al., 1992; McCarty et al., 1993).[4]

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[4] Based on the three-state model, the mean dwell time in the intermediate closed state is $(k_{open} + k_{off})^{-1}$ and the frequency of these brief events per opening is k_{open}/k_{off}. It is possible to use

The three-state model can be modified to account for both of these states and provide a more complete description of CFTR gating:

$$C_0 \rightleftharpoons ATP + C_1 \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} ATP\text{-}C_2 \overset{k_{open}}{\underset{k_{close}}{\rightleftharpoons}} ATP\text{-}O \rightleftharpoons ATP\text{-}C_3 \qquad (S2a)$$

C_0 is the dephosphorylated state, and $ATP\text{-}C_3$ represents the brief closed state. The remaining states correspond to the three-state mechanism shown in scheme S1, and the kinetic constants have been defined previously. This scheme is similar to the model for CFTR gating proposed by Haws et al. (1992), and it is based on the presumption that $ATP\text{-}C_3$ can only undergo transitions to $ATP\text{-}O$. However, as noted by Sakmann and Trube (1984), we cannot exclude the possibility that $ATP\text{-}C_3$ is interposed between $ATP\text{-}C_2$ and $ATP\text{-}O$:

$$C_0 \rightleftharpoons ATP + C_1 \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} ATP\text{-}C_2 \overset{k_{C2\text{-}3}}{\underset{k_{C3\text{-}2}}{\rightleftharpoons}} ATP\text{-}C_3 \rightleftharpoons ATP\text{-}O \qquad (S2b)$$

$k_{C2\text{-}3}$ and $k_{C3\text{-}2}$ are the rate constants for the transitions between $ATP\text{-}C_2$ and $ATP\text{-}C_3$.

The rate of dephosphorylation was relatively slow, and these events were excluded from the current and fluctuation-derived measurements. However, by varying the conditions, it may be possible to alter the phosphorylation or dephosphorylation rate and resolve two low-frequency Lorentzians in the PDS and/or two closed times in the event duration histogram. Under these circumstances, it may be possible to analyze the two opening rates and obtain estimates of the phosphorylation and dephosphorylation rates (Hwang, Horie, and Gadsby, 1993). Since both opening rates are expected to be ATP sensitive, an understanding of the three-state mechanism is required to quantify the phosphorylation-dephosphorylation transitions.

Possible events resulting from transitions to $ATP\text{-}C_3$ were excluded from our analysis either by the low-pass filter or by limitations imposed by system noise. Furthermore, the ability to describe 98% of the change in P_o using a single set of transitions provides some justification for excluding $ATP\text{-}C_3$ from analysis. However, an increase in the duration or frequency of these events will cause a marked reduction in P_o, and this alternative hypothesis may also explain some CF-causing point mutations (Anderson and Welsh, 1992a; Sheppard, Rich, Ostedgaard, Gregory, Smith, and Welsh, 1993). There is some preliminary evidence for higher frequency Lorentzian components in whole-cell recordings of CFTR-transfected Sf9 cells (Larsen, Fullton, Stutts, Boucher, and Price, 1992) and in cell-attached patches of CFTR-transfected 3T3 cells (Fischer and Machen, 1993). Our results show that the three-state mechanism can not account for frequent closures $\ll 185 \pm 18$ ms and provides a mechanistic interpretation of these events (i.e., $ATP\text{-}C_3$ in S2) that can be tested in future study.

these relationships and the estimate of opening rate (5.4 s^{-1}) to calculate the predicted frequency of 3 ms closed events: $5.4/((0.003)^{-1} - 5.4) = 0.016$. Therefore, <1 in 50 openings is expected to contain a brief closed event. This calculation also illustrates why bursting behavior will appear to be absent when k_{open} is rate limiting.

Finally, in addition to modifying the three-state gating mechanism to include a dephosphorylated closed state and a brief closed state, we must also consider the possibility of multiple binding reactions. As mentioned previously, the genetic sequence for CFTR contains two regions that are thought to code for NBF's. In this regard, it is important to note that the single-site, three-state model is consistent with a number of ligand-activated channels that possess two equivalent binding sites such as the acetylcholine receptor channel (del Castillo and Katz, 1957; Maelicke, 1992), the γ-aminobutyric acid receptor channel (Sakmann, Hamill, and Bormann, 1983; Bormann and Clapham, 1985), and an extracellular ATP-activated nonselective channel (Bean, 1990, 1992). Likewise, the effect of ATP on CFTR open probability and current fluctuations suggests that if multiple binding reactions are present, then they appear as a single set of transitions under the experimental conditions employed in our study. This hypothesis is also consistent with the observation that k_{open} is rate limiting (see Results). However, it may be possible to separate binding events by varying the experimental conditions. Anderson and Welsh (1992a, 1992b) present evidence for multiple nonequivalent ATP binding sites. They report a nonlinear Eadie-Hofstee plot of the effect of ATP on P_o that has two possible interpretations: (a) multiple sites with different affinity that increase P_o independently or (b) negative cooperativity.[5] Further study is needed to address the intriguing possibility that the ATP binding sites may be sensitive to experimental conditions. Finally, the effect of low ATP concentrations may reveal multiple binding reactions, and additional investigation is also needed to test this hypothesis (Bean, 1990).

Significance of the Apparent Dissociation Constant for ATP

Our results provide an estimate of k_{off}/k_{on} (44 ± 14 μM) that is considerably lower than measures of total cellular ATP that have been reported to vary between 3 and 6 mM (Kashket 1982; Ashcroft, Weersinghe, and Randle, 1973). This discrepancy is likely to raise some concern regarding the physiological significance of ATP binding to CFTR. It is of interest to note that similar concerns were raised regarding the periplasmic histidine transporter (K_m = 200 μM; Ames, Nikaido, Groarke, and Petithory, 1989) and ATP-sensitive K channels (K_{ATP}) in pancreatic β cells (K_m = 15–50 μM, Cooke and Hales, 1984). There are two lines of evidence that reconcile the difference between these apparent dissociation constants and the measures of total cellular ATP. First, digitonin permeabilization has revealed that cytoplasmic ATP is ~0.5 mM, and it suggests that ATP is compartmentalized within cells (Malaisse and Sener, 1987). Second, measures of cell-attached K_{ATP} channel activity in digitonin- or saponin-permeabilized cells revealed that the concentration–response curve was shifted fivefold to the right (Kakei, Kelly, Ashcroft, and Ashcroft, 1985; Dunne, Findlay, Petersen, and Wollheim, 1986). This result implicated an additional intracellular modulatory factor that was later identified as ADP (Kakei et al., 1986; Dunne and Peterson, 1986).

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[5] These two possibilities cannot be separated because of the narrow range of the concentration–response relationship and the inability of the Eadie-Hofstee plot to properly analyze multiple-site data (Wilkinson, 1961; Munson and Rodbard, 1983).

Presumably, ATP compartmentalization and additional intracellular modulatory factors also reconcile the difference between the dissociation constant for ATP and the measures of total cellular ATP. There are data to support the possibility of an additional modulator since, the ATP response of CFTR in *Staphlococcus aureus* α-toxin–permeabilized sweat ducts and T84 colonocytes was shifted to the right (Quinton and Reddy, 1992; Bell and Quinton, 1993). Furthermore, ADP reduces the activity of CFTR and shifts the ATP concentration–response curve to the right (Anderson and Welsh, 1992a). We have obtained preliminary evidence that supports this notion, quantifies ADP-induced alterations in CFTR noise, and suggests that ADP decreases the opening rate (Schultz, Venglarik, Bridges, and Frizzell, 1993). Thus, the ATP dissociation constant should be interpreted in the context of a more complete regulatory scheme.

APPENDIX

Derivation of the Concentration–Response Relationship for P_o from the Three-state Model

The three-state mechanism can be used to derive the relationship between ATP concentration and P_o in terms of the kinetic constants. Based on this simple model, single-channel records are expected to contain openings punctuated by relatively brief closures (or bursts) alternating with longer-lived, ligand-sensitive closures (for review see Colquhoun and Hawkes, 1982, 1983). Therefore, the ability of a channel to conduct ions depends on two conditions: (*a*) that the channel is bursting, and (*b*) the channel is open within the burst. P_o is simply the product of the two probabilities:

$$P_o = (P_o)_{burst}(P_{burst}) \tag{9}$$

$(P_o)_{burst}$ is the probability of being open within a burst and P_{burst} is the probability of being in a burst. This equation efficiently separates the ATP-sensitive aspect of gating (P_{burst}) from the ATP-insensitive fluctuations $[(P_o)_{burst}]$. In addition, $(P_o)_{burst}$ provides a model-independent description of gating behavior within bursts that can allow this simple derivation to be applied to more complicated bursting schemes, such as those expected for ATP-C_3 shown in S2. Based on the three-state mechanism, the probability that a channel is bursting is:

$$P_{burst} = r_+/(r_+ + r_-) \tag{10}$$

r_+ is the exit rate from the ATP-dependent closed time, and r_- is the exit rate from the burst. These rates have been previously defined by Eqs. 4 and 5b. Substitution of Eq. 4 into Eq. 10 and rearranging the result yields:

$$P_{burst} = \frac{\dfrac{k_{open}}{k_{open} + r_-}[ATP]}{\dfrac{r_-}{k_{open} + r_-} \cdot \dfrac{k_{off}}{k_{on}} + [ATP]} \tag{11}$$

This relation has the form of a Michaelis-Menten function. The maximum probability of being in a burst $[(P_{burst})_{max}]$ is $k_{open}/(k_{open} + r_-)$, which agrees with the expected

result obtained by substituting k_{open} for r_+ in Eq. 10. The Michaelis constant is the product of the dissociation constant (k_{off}/k_{on}) and $r_-/(k_{open} + r_-)$, which corresponds to $[1 - (P_{burst})_{max}]$. Eq. 9 shows that P_o can be obtained by multiplying Eq. 11 by the probability of being open within a burst $[(P_o)_{burst}]$:

$$P_o = \frac{(P_o)_{burst} \frac{k_{open}}{k_{open} + r_-} [ATP]}{\frac{r_-}{k_{open} + r_-} \cdot \frac{k_{off}}{k_{on}} + [ATP]}$$ (12)

Therefore, P_o is also expected to vary as a Michaelis-Menten function of ATP concentration. Note that $(P_o)_{max}$ is the product of $(P_o)_{burst}$ and $(P_{burst})_{max}$ (i.e., $k_{open}/(k_{open} + r_-)$) as predicted by Eq. 9. The K_m is identical to that shown in Eq. 11. Substituting $(P_o)_{max}$ for $(P_o)_{burst}$ $k_{open}/(k_{open} + r_-)$ and a constant (R) for $r_-/(k_{open} - r_-)$ provides a more general relation that was cited previously without derivation:

$$P_o = \frac{(P_o)_{max}[ATP]}{R \cdot \frac{k_{off}}{k_{on}} + [ATP]}$$ (3)

Although Eqs. 3, 11, and 12 conform to the formula derived by Michaelis and Menten (1913), the Michaelis constant is not the same as the dissociation constant for ATP. This distinction raises an important caveat regarding the use and interpretation of the Michaelis constant for CFTR open probability. However, based on the three-state model, it is possible to relate the K_m to k_{off}/k_{on} using Eq. 12 and the estimates of the kinetic constants.

Note added in proof. The three-state activation mechanism used herein to describe the relationship between ATP binding and CFTR opening is not analogous to the three-state scheme recently proposed by Winter, Sheppard, Carson, and Welsh (1994). Specifically, we provide evidence that there are two closed states underlying the long-lived ATP-sensitive closed time based on a detailed analysis of the relationship between ATP concentration and CFTR gating. Thus, it is necessary to divide "C_1" in the scheme proposed by Winter et al. (1994) into two states and to note that "β_1" does not provide a measure of an ATP/ADP dependent rate constant. The units assigned to "β_1" by Winter et al. (1994) also indicate that it is a transition rate since a ligand dependent rate constant must possess units of concentration (i.e., $M^{-1}s^{-1}$).

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