Effect of ATP Concentration on CFTR Cl^- Channels: A Kinetic Analysis of Channel Regulation

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ABSTRACT Phosphorylated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels require nucleoside triphosphates, such as ATP, to open. As the concentration of intracellular ATP increases, the probability of the channel being open (P_o) increases. To better understand how ATP regulates the channel, we studied excised inside-out membrane patches that contained single, phosphorylated CFTR Cl^- channels and examined the kinetics of gating at different concentrations of ATP. As the ATP concentration increased from 0.1 to 3 mM the mean closed time decreased, but mean open time did not change. Analysis of the data using histograms of open- and closed-state durations, the maximum likelihood method, and the log-likelihood ratio test suggested that channel behavior could be described by a model containing one open and two closed states ($C_1 \leftrightarrow C_2 \leftrightarrow O$). ATP regulated phosphorylated channels at the transition between the closed states C_1 and C_2: as the concentration of ATP increased, the rate of transition from C_1 to C_2 ($C_1 \rightarrow C_2$) increased. In contrast, transitions from C_2 to C_1 and between C_2 and the open state (O) were not significantly altered by ATP. Addition of ADP in the presence of ATP decreased the transition rate from C_1 to C_2 without affecting other transition rates. These data suggest that ATP regulates CFTR Cl^- channels through an interaction that increases the rate of transition from the closed state to a bursting state in which the channel flickers back and forth between an open and a closed state (C_2). This transition may reflect ATP binding or perhaps a step subsequent to binding.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989) is a Cl^- channel that requires both phosphorylation, usually by the cAMP-dependent protein kinase (PKA), and the presence of intracellular nucleoside triphosphates such as ATP to open (for reviews see Riordan, 1993; Welsh et al., 1992). Phosphorylation of the channel occurs on the R domain, a unique domain containing a number of consensus phosphorylation sites (Riordan et al., 1989; Cheng et al., 1991). In phosphorylated channels, ATP increases the single channel open-state probability of PKA phosphorylated channels in a dose-dependent manner with half-maximal activity at about 270 μM (Anderson et al., 1991a). Two lines of evidence indicate that ATP interacts with the nucleotide-binding domains (NBDs) in CFTR Cl^- channels. First, the relationship between ATP concentration and channel activity is altered in CFTR containing site-directed mutations in the NBDs (Anderson et al., 1991a; Anderson and Welsh, 1992). Second, nucleotide analogs have been shown to interact directly with membrane-associated CFTR (Travis et al., 1993) and with sequences within the NBDs (Hartman et al., 1992; Thomas et al., 1991; Ko et al., 1993).

However, we have little understanding of how ATP interacts with the NBDs to regulate CFTR Cl^- channel activity. This issue is particularly important in that many cystic fibrosis-associated mutations occur in the NBDs (Tsui, 1992). To better understand how ATP regulates the CFTR Cl^- channel, we examined the effect of ATP on single CFTR Cl^- channels and analyzed the results using a simple kinetic model.

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MATERIALS AND METHODS

Cells and CFTR expression

Two different expression systems were used for these studies. CFTR was transiently expressed in HeLa cells using a *vaccinia* virus-T7 hybrid expression system as previously described (Rich et al., 1990; Elroy-Stein et al., 1989). CFTR was also expressed in NIH-3T3 fibroblasts that had been transfected stably with a retrovirus expressing human wild-type CFTR (Anderson et al., 1991b). We could not detect a difference between the expression systems, and, therefore, the data were combined.

Patch clamp technique

The excised, inside-out patch-clamp technique was used to record single channel currents (Hamill et al., 1981; Sheppard et al., 1993). The pipette solution contained (in mM): 140 N-methyl-D-glucamine, 100 aspartic acid, 5 $CaCl_2$, 2 $MgSO_4$, and 10 N-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES), pH 7.3 with HCl ($[Cl^-]$ = 54 mM). The bath contained: 140 N-methyl-D-glucamine, 3 $MgCl_2$, 1 Cs-EGTA and 10 TES, pH 7.3 with HCl ($[Cl^-]$ = 146 mM). The concentration of ATP in the bath varied within an experiment, as indicated. All experiments were performed at 34–36°C. After excision of patches, channels were activated by the addition of the catalytic subunit of PKA (~75 nM, Promega Corp., Madison WI) in the presence of 1 mM ATP. PKA was subsequently washed out of the bath and was absent during the remainder of the experiment. We analyzed data only from patches that contained a single active channel. We determined that a patch contained only a single channel if the simultaneous opening of two channels was not observed during the course of an experiment, even at the

highest concentrations of ATP (3 mM). An experiment typically lasted 15–45 min, during which 3 mM ATP was present in the bathing solution for 3–8 min.

Single channel currents were initially recorded on videotape using a PCM-2 A/D VCR adapter (Medical Systems Corp. Greenvale, NY) and either an Axopatch-1D (Axon Instruments, Foster City, CA) or a List-Medical EPC-7 (Adams and List Associates, Ltd., Westbury, NY) amplifier for voltage clamping and amplification of current. On playback, records were filtered with an eight-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) at a corner frequency of 1 kHz. A microcomputer (Apple Macintosh, Apple Computer, Inc., Cupertino, CA) equipped with an multifunctional data acquisition board (NB-MIO-16) and LabVIEW 2 software (National Instruments, Austin, TX) was used to digitize the data at a sampling rate of 5 kHz. The data were then digitally filtered at a corner frequency of 500 Hz, after which a table of open and closed times was created using a half-amplitude crossing criterion for event detection. Single channel open-state probability was calculated by counting the number of samples above the half-amplitude threshold and dividing by the total number of samples in the record.

Histogram analysis

Open- and closed-time histograms were created with a lower limit of 1.0 ms and upper limits of 100 ms and 1 s, respectively. This lower limit was applied to limit the effects of system dead time (<0.8 ms) For open times the bin width was 5 ms, and for closed times it was 50 ms. The use of other combinations of ranges (1–10 ms for lower limit, 0.1–2 s. for upper limit) and bin widths (1–50 ms) yielded qualitatively similar results (data not shown). Unweighted histograms were fit using a single exponential and the sum of two and three exponentials using the nonlinear Levenberg-Marquardt method in LabVIEW 2 to determine the least squares set of coefficients in each case. To determine if a sum of exponential components was significantly better than a fit with fewer components, the residual sum of squares was compared as previously described (Rao, 1973; Mannervik, 1982; Horn, 1987). The improvement of the residual sum of squares found in the more complex fit is weighted by the number of samples and by the difference in the number of parameters between the two models to determine an F-statistic, which can be tested at a given significance level (typically $p < 0.05$). Logarithmic histograms also were created using 8–12 bins per decade and a square root ordinate. This allows a quick estimation of the number of exponential components within the distribution based on the number of observed peaks (Sigworth and Sine, 1987).

Maximum likelihood analysis

The use of the maximum likelihood method to determine the kinetics of ion channel behavior has been extensively described (Colquhoun and Sigworth, 1983; Ball and Sansom, 1989; Colquhoun and Hawkes, 1981; Horn and Lange, 1983). To derive the open- and closed-time probability density functions for the models, we made use of the symbolic algebra program Maple V (Waterloo Maple Software, Waterloo, Ontario, Canada) to symbolically solve the matrix equations in terms of the rate constants. The resulting equations were equivalent to those previously reported (Colquhoun and Sigworth, 1983; Horn and Lange, 1983) and were transferred into LabVIEW 2 to determine the set of rate constants that yielded the maximum likelihood for the observed open and closed times. The log-likelihood ratio test (Akaike, 1974) and its application in patch-clamp experiments has been described previously (Horn, 1987). This test discriminates between models based on the logarithm of the ratio of the maximum likelihoods determined for the two competing models. This method can also be used to "penalize" a more complex model based on the difference in the degrees of freedom between the models. The significance of the improvement can then be tested at a specified significance level.

Statistical methods

To test for differences between groups of interventions, a standard analysis of variance (ANOVA) was used. In cases where a difference was detected between groups, a Spearman correlation test was performed. To compare only two sets of data a Student's t-test was performed. Differences were considered statistically significant when $p < 0.05$. All tests were performed using the StatWorks software package (Cricket Software, Philadelphia, PA).

RESULTS AND DISCUSSION

Effect of ATP on mean open and closed times

We previously showed that increasing the concentration of ATP on the cytosolic surface of the membrane patch increased the open-state probability (P_o) (Anderson and Welsh, 1992). This could result from an increase in the mean open time, a decrease in the mean closed time, or a combination of both. To discriminate between these possibilities, we studied excised patches that contained only a single CFTR Cl^- channel and varied the concentration of ATP that bathed the cytosolic surface of the patch. Fig. 1 shows an example. Interventions were repeated within a single experiment, as conditions allowed, to ensure stability of the experimental preparation in terms of the kinetic parameters measured. In the experiments reported here, no rundown was observed as determined by comparing P_o's at the same ATP concentrations at different times within an experiment.

After creating a list of open and closed times for each intervention, the mean open and closed times were calculated and plotted as a function of ATP concentration (Fig. 2). To test for a difference in mean open times, a one-way ANOVA was performed. This test indicated that there was no significant difference between the groups at the different ATP concentrations ($p = 0.22$). From this we conclude that the mean open time is not affected by changes in ATP concentration.

A similar test was performed on the mean closed times, and a significant difference was found between groups ($p = 0.04$). The test of statistical significance (ANOVA) only indicates that one or more of the groups is different from the others and gives no indication of a relationship between ATP concentration and mean closed time. To test for such a relationship, a Spearman correlation test was performed. This test is limited in that it is based upon a linear correlation, thus

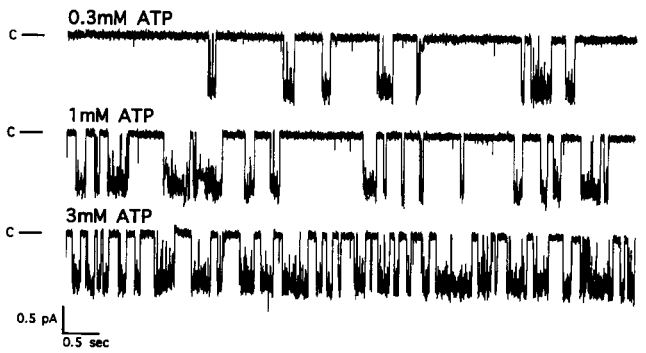

FIGURE 1 Single-channel currents recorded from an excised patch. The bath (cytosolic) solution contained the indicated concentration of ATP. Downward deflections represent channel openings. The holding potential is −120 mV with a bandwidth of 500 Hz.

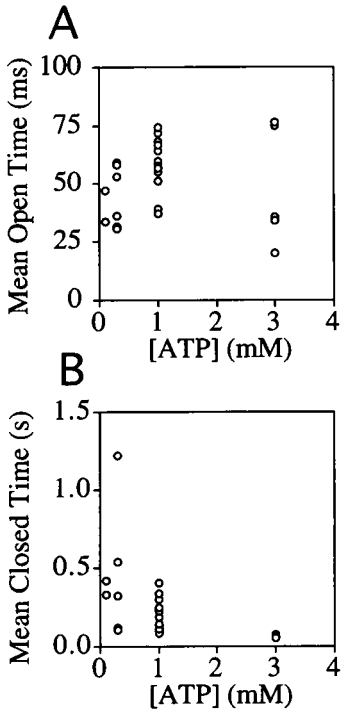

FIGURE 2 Effect of ATP concentration on mean open (A) and closed times (B). Mean dwell times were calculated at 0.1, 0.3, 1.0, and 3.0 mM ATP. Mean open times do not change with different ATP concentration ($p < 0.05$), whereas mean closed times are inversely related to ATP concentration ($p < 0.05$). Each symbol represents an individual determination. Data are summarized in Table 1.

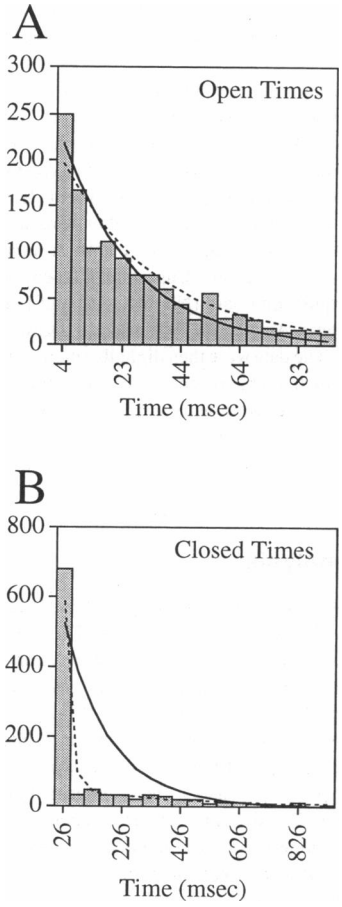

FIGURE 3 Channel open (*A*)- and closed (*B*)-time histograms at 0.3 mM ATP. These representative histograms are from a single intervention in one experiment. They were fit with a single exponential curve (*solid lines*) as well as the sum of two exponential curves (*dashed lines*). The open-time distribution (*A*) is best fit with a single exponential curve ($p = 0.05$). The closed-time distribution is fit significantly better by the sum of two, but not three, exponential curves ($p = 0.05$).

it will underestimate the level of statistical significance in the case of a nonlinear relationship. However, the result indicates a significant, negative correlation between ATP concentration and mean closed time ($p < 0.001$). This result suggests that an increase in ATP concentration leads to a decrease in mean closed time. These data suggest that the rate at which the channel opens, but not the rate at which it closes, depends on the concentration of ATP. This interpretation is consistent with the notion that the interaction of ATP with the channel is a rate-limiting step in channel opening.

Open and closed time decay rates

We analyzed histograms of open and closed times to determine if there are more than one open or closed states (Fig. 3). We assessed the number of exponentially decaying components necessary to best fit the histograms for both open and closed times at each intervention. Open time distributions were well fit by a single exponential curve; the sum of two exponential curves did not produce a significantly better fit. Histograms of the closed-time durations were fit significantly better by a sum of two exponential curves than by a single exponential. The sum of three exponential curves did not produce a significantly better fit (not shown). These results suggest a minimal model of ATP regulation containing one open and two closed states. To our knowledge, the only published data that addressed the gating kinetics of wild-type CFTR is from channels in immortalized human airway cells (Haws et al., 1992). Using histograms of data from multichannel patches, they estimated that a single open state and a minimum of two closed states were necessary to describe the kinetics of phosphorylated channels.

There are several limitations in using open and closed time histograms to describe channel kinetics. The first is that the exponential fit is strongly influenced by the parameters used to create the histogram, namely bin width and the number of bins used in the fit. The fit also is biased, because a bin containing very few long dwell times is given the same weight as a bin containing hundreds of short dwell times. These factors become increasingly important when the data sets are relatively small (1000–3000 events in our studies). One final limitation in histogram analysis is that, in cases where there are more than one open or closed state, the decay rates do not have a simple relationship to the rate constants used to describe transitions between states (Colquhoun and

Sigworth, 1983). To address these limitations we used the maximum likelihood method to analyze the data.

Model discrimination

We considered the three possible models that contain one open state and two closed states, as well as a basic two-state model containing a single open and closed state. Open and closed times were fit to the four different models using the maximum likelihood method. In general, a more complex model (which contains simpler models as subsets) will always fit the data better, because it will have more degrees of freedom. Therefore, to determine whether a more complex model fit significantly better than a simple model, we used the log-likelihood ratio test, which penalizes the more complex model based on the difference in the degrees of freedom between the two models.

We found that for every ATP concentration, the rank order for the highest likelihood was:

$$\begin{matrix} C_2 \leftrightarrow O \\ \nwarrow \quad \nearrow \\ C_1 \end{matrix} > C_1 \leftrightarrow C_2 \leftrightarrow O = C_1 \leftrightarrow O \leftrightarrow C_2 > C_1 \leftrightarrow O \quad (1)$$

When the complexity of the model was taken into account, the $C_1 \leftrightarrow C_2 \leftrightarrow O$ model fit the data significantly better than the simpler $C_1 \leftrightarrow O$ model in all cases ($p < 0.05$). However, the general three-state model (the model positioned to the left in equation 1) was not significantly better than either of the linear three-state models ($p < 0.05$). The $C_1 \leftrightarrow C_2 \leftrightarrow O$ and $C_1 \leftrightarrow O \leftrightarrow C_2$ models yield the same maximum likelihood values, thus we are unable to discriminate between them using this criterion. Therefore, we conclude that a linear three-state model is sufficient to describe the kinetics of phosphorylated channels. Note, however, that an additional closed state would be required to describe the transition from the closed, dephosphorylated state to a state C_1 or C_2 at which the channel was available for opening in response to ATP.

The strength of the conclusion is dependent on two important factors. The first is the limited duration of the experimental interventions (2–5 min for each intervention). It is possible that given a much longer recording, the more complex, general three-state model might become significantly better. The second factor is that we did not test more complex models containing more than one open state and more than two closed states. Although histogram analysis suggested that a three-state model was sufficient, it is possible that other states are "hidden" within the histograms due to the limitations of that method and the finite data set. Even using the maximum likelihood method, prohibitively long recordings would be required to distinguish differences between models. This can be seen by assuming a third closed state, resulting in a $C_1 \leftrightarrow C_2 \leftrightarrow C_3 \leftrightarrow O$ model. The first closed state might be visited rarely and therefore be represented in the measured closed times at a very low frequency. In interpreting how the model represents actual channel behavior, the transition from one state to a neighboring state may represent several steps that we are unable to discriminate at the present time.

Effect of ATP concentration on rate constants

To determine how ATP regulates CFTR Cl^- channels, we examined the effect of ATP concentration on the rate constants given by:

$$C_1 \underset{\alpha_1}{\overset{\beta_1}{\rightleftharpoons}} C_2 \underset{\alpha_2}{\overset{\beta_2}{\rightleftharpoons}} O \quad (2)$$

where β_1, β_2, α_1, and α_2 are the transition rates between states, given in s^{-1}. We chose this model as a starting point rather than the $C_1 \leftrightarrow O \leftrightarrow C_2$ model, because it is similar to the models proposed for other agonist-gated channels. The rate constants determined using the maximum likelihood method are shown in Fig. 4, and mean values are given in Table 1. Examination of the values for α_2 shows that it does not change at the different ATP concentrations. This is confirmed by an ANOVA, which indicates that there is no significant ($p = 0.24$) difference between the groups of values at the different ATP concentrations. Because α_2 is the only rate constant that determines the open-time distribution, this result is consistent with the data showing that mean open time is not a function of ATP concentration (Fig. 2). Rate constants β_2 and α_1 were also independent of ATP concentration ($p > 0.4$). In contrast, there was a significant difference in the values for β_1 between the different ATP concentrations tested ($p < 0.001$). A correlation test found a significant, positive correlation between ATP concentration and β_1 ($p < 0.001$). We conclude from this result that ATP only affects the transition from C_1 to C_2, and that the other transitions are independent of ATP concentration.

The effect of ATP concentration on the rate constants (Fig. 4, Table 1) explains the observed behavior of phosphorylated CFTR Cl^- channels. The channel manifests a bursting behavior; when an open channel closes to the short-lived closed

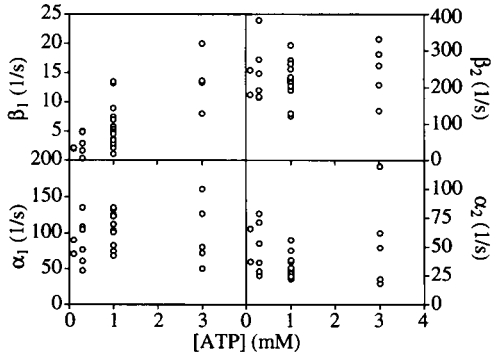

FIGURE 4 Effect of ATP concentration on rate constants determined by the maximum likelihood fit to the $C_1 \leftrightarrow C_2 \leftrightarrow O$ model. α_2, β_2, and $\alpha 1$ are independent of ATP concentration ($p > 0.05$), whereas β_1 has a significant correlation with ATP concentration ($p < 0.05$). Each symbol represents an individual determination. Data are summarized in Table 1.

TABLE 1 Average dwell times and rate constants

	0.1 mM ATP	0.3 mM ATP	1.0 mM ATP	3.0 mM ATP	1 mM ATP + 1 mM ADP	AVE
n	2	6	12	5	3	28
P_o	0.098 ± 0.03	0.142 ± 0.04	0.262 ± 0.03	0.441 ± 0.06	0.075 ± 0.03	n.a.*
t_o (ms)	40 ± 9	45 ± 6	58 ± 3	48 ± 13	58 ± 6	52 ± 2
t_c (ms)	377 ± 65	438 ± 186	194 ± 28	56 ± 4	826 ± 246	n.a.
α_1 (l/s)	80.2 ± 14	88.9 ± 15	106.2 ± 6	97.9 ± 22	91.3 ± 5	100.2 ± 4.6
β_1 (1/s)	2.05 ± 0.1	3.01 ± 0.8	6.02 ± 1.0	15.02 ± 2.5	1.22 ± 0.5	n.a.
α_2 (1/s)	51.7 ± 21	49.1 ± 10	31.4 ± 3	54.4 ± 21	29.0 ± 3	41.5 ± 3
β_2 (1/s)	214.0 ± 48	239.8 ± 36	218.8 ± 14	245.2 ± 38	181.2 ± 6	219.6 ± 9

The table shows mean values ± SE calculated for several experimentally derived parameters for each experimental intervention. Average values across all interventions are given where applicable (no significant difference between groups).
* n.a., not applicable.

state (C_2), it is most likely to return quickly to O, because β_2 is a faster rate constant than α_1. However, once the channel makes the transition from C_2 to the long-lived closed state (C_1) the rate of transition back to C_2 is relatively slow. This gives the appearance of bursts of channel openings that are interrupted by brief flickers to the closed state C_2. The bursts of channel openings are separated by sojourns to the closed state C_1. This is apparent from an examination of Fig. 1. As the ATP concentration increases, the interval between bursts of openings is shortened (as β_1 increases) with no apparent change in the duration of bursts or in the closed times within bursts (C_2).

In contrast, when we used the $C_1 \leftrightarrow O \leftrightarrow C_2$ model, instead of the $C_1 \leftrightarrow C_2 \leftrightarrow O$ model, the data indicated that ATP altered three of the four rate constants instead of just one. Thus an argument based on parsimony would favor the $C_1 \leftrightarrow C_2 \leftrightarrow O$ model over the $C_1 \leftrightarrow O \leftrightarrow C_2$ model.

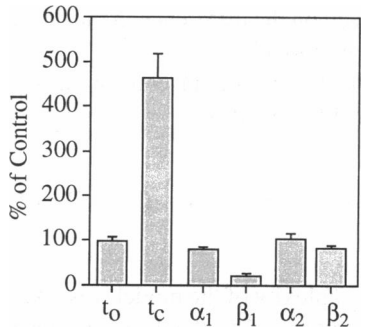

FIGURE 5 Effect of ADP on single channel kinetics. Calculated values for mean open (t_o) and closed (t_c) times, α_1, α_2, β_1, and β_2 are shown as a percentage of control values. Under control conditions the bath contained 1 mM ATP, and during the experimental condition the bath contained 1 mM ATP plus 1 mM ADP. t_c and β_1 are the only values that were significantly different from control values (Student's-t test, $p < 0.05$). Mean values are given in Table 1. ($n = 3$)

Effect of ADP on rate constants

We previously showed that ADP inhibited ATP-supported channel activity (Anderson and Welsh, 1992). Addition of 1 mM ADP to a bath (cytosolic) solution containing 1 mM ATP significantly increased the mean closed time with no significant effect on mean open time (Fig. 5 and Table 1). Examination of the derived rate constants indicates that α_1, α_2, and β_2 were not significantly altered, but β_1 decreased ($p < 0.05$).

CONCLUSION AND SPECULATION

Our data indicate that the activity of phosphorylated CFTR Cl^- channels can be described by a model containing at least three states: $C_1 \leftrightarrow C_2 \leftrightarrow O$. ATP increases and ADP decreases single channel open-state probability by altering the transition rate, β_1, from C_1 to C_2. Our data do not allow us to determine the nature of the interaction between ATP and the channel that determines β_1. The value of β_1 that we measured, 15 s^{-1} (at 3 mM ATP), is relatively slow and is in the range of turnover rates for ATP hydrolysis by the Na^+-K^+-ATPase (130–180 ATP/s, (Jorgensen, 1975)) and the sarcoplasmic reticulum Ca^{2+}-ATPase (80–180 ATP/s; MacLennan et al., 1971; Bannerjee et al., 1979). However, β_1 could be controlled by ATP binding or by one or more steps subsequent to ATP binding. Such a step could be a conformational change resulting from hydrolysis of ATP, a nonhydrolytic conformational change, or a sequence of conformational changes. Our observation that β_1 is not zero when ATP concentration is extrapolated to zero suggests that β_1 does not solely represent the association or binding of ATP to the channel.

Our data also indicate that ADP inhibits the channel at the same kinetic step, β_1, that was affected by ATP. One simple interpretation of this result is that ADP competes with ATP for binding and/or activation. This result is consistent with previous results showing that ADP inhibits binding of the ATP analog 8-azidoadenosine 5′-tri-phosphate to membrane-associated CFTR (Travis et al., 1993).

Finally, the kinetic model shows that the step from C_2 to C_1, which would inactivate the channel, seems to be independent of ATP. Moreover, the transitions between C_2 and O were independent of ATP. Thus, we speculate that conformational changes in the protein that are responsible for the duration of bursts of openings (predominately determined by α_1) and transitions between C_2 and O within bursts (determined by β_2 and α_2) may not be dependent on ATP

concentration. Alternatively, these steps may be dependent on ATP in an all-or-none manner (or at least a much lower concentration than we used) and thus would change little as the concentration of ATP was altered.

These data begin to provide some insight into how intracellular ATP regulates the CFTR Cl^- channel. Additional insights will require the combination of functional and biochemical studies to elucidate how ATP interacts with the NBDs of CFTR to control the molecular transitions between the open and closed states.

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