Regulation of CFTR Cl⁻ Channel Gating by ADP and ATP Analogues

BRUCE D. SCHULTZ, CHARLES J. VENGLARIK, ROBERT J. BRIDGES, and RAYMOND A. FRIZZELL

From the Department of Physiology and Biophysics and Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

ABSTRACT   The cystic fibrosis gene product (CFTR) is a chloride channel which, once phosphorylated, is regulated by nucleotide phosphates (Anderson, M. P., and M. J. Welsh. 1992. *Science*. 257:1701–1704; Venglarik, C. J., B. D. Schultz, R. A. Frizzell, and R. J. Bridges. 1994. *Journal of General Physiology*. 104:123–146). Nucleotide triphosphates initiate channel activity, while nucleotide diphosphates and nonhydrolyzable ATP analogues do not. To further characterize the role of these compounds on CFTR channel activity we examined their effects on chloride channel currents in excised inside-out membrane patches from CFTR transfected mouse L cells. ADP competitively inhibited ATP-dependent CFTR channel gating with a K_i of 16 ± 9 μM. AMP neither initiated CFTR channel gating nor inhibited ATP-dependent CFTR channel gating. Similarly, ATP analogues with substitutions in the phosphate chain, including AMPCPP, AMPPCP, AMPPNP, and ATPγS failed to support CFTR channel activity when present at the cytoplasmic face of the membrane and none of these analogues, when present at three to 10-fold excess of ATP, detectably altered ATP-dependent CFTR channel gating. These data suggest that none of these ATP analogues interact with the ATP regulatory site of CFTR which we previously characterized and, therefore, no inference regarding a requirement for ATP hydrolysis in CFTR channel gating can be made from their failure to support channel activity. Furthermore, the data indicate that this nucleotide regulatory site is exquisitely sensitive to alterations in the phosphate chain of the nucleotide; only a nonsubstituted nucleotide di- or triphosphate interacts with this regulatory site. Alternative recording conditions, such as the presence of kinase and a reduction in temperature to 25°C, result in a previously uncharacterized kinetic state of CFTR which may exhibit distinctly different nucleotide dependencies.
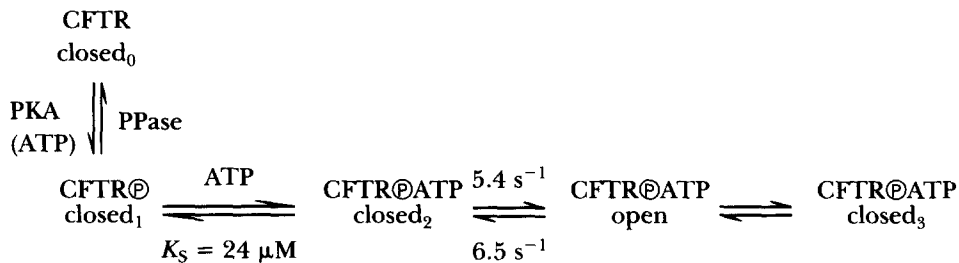
INTRODUCTION

The gene which, when mutated, causes cystic fibrosis has been isolated, cloned, sequenced, and named the cystic fibrosis transmembrane conductance regulator (CFTR; Riordan, Rommens, Kerem, Alon, Rozmahel, Grzelczak, Zielenski, Lok, Plavsic, Chou, Drumm, Iannuzzi, Collins, and Tsui, 1989). The CFTR gene product is a member of the traffic ATPase or ATP binding cassette (ABC) family of proteins

which includes prokaryotic permeases, the STE6 protein of yeast, and the multidrug resistance protein (MDR1) of humans (Riordan et al., 1989; Hyde, Emsley, Hartshorn, Mimmack, Gileadi, Pearce, Gallagher, Gill, Hubbard, and Higgins, 1990; Ames and Lecar, 1992). Structural homology is seen throughout this family in that all proteins consist of two putative membrane-spanning domains (MSDs) and two putative nucleotide-binding domains (NBDs; Hyde et al., 1990; Ames and Lecar, 1992). CFTR differs from other members of the ABC family in that it also includes a regulatory domain (R domain) which contains multiple consensus phosphorylation sites for both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC; Riordan et al., 1989; Rich, Gregory, Anderson, Manavalan, Smith, and Welsh, 1991; Picciotto, Cohn, Bertuzzi, Greengard, and Nairn, 1992). Most ABC proteins are presumed to hydrolyze ATP in transporting solutes across the cell membrane as has been demonstrated for the periplasmic permeases (Hyde et al., 1990; Ames and Lecar, 1992). However, CFTR is a chloride channel which requires both PKA-dependent phosphorylation to initiate activity and the continued presence of ATP for steady state channel gating (Anderson, Berger, Rich, Gregory, Smith, and Welsh, 1991; Nagel, Hwang, Nastiuk, Nairn, and Gadsby, 1992; Venglarik, Schultz, Frizzell, and Bridges, 1994; see reviews by Anderson, Sheppard, Berger, and Welsh, 1992; Collins, 1992). It has been suggested that CFTR hydrolyzes ATP as a necessary step in channel gating. The evidence for this derives from the absence of channel activity in the presence of nonhydrolyzable ATP analogues (Anderson et al., 1991) and the effects of reduced temperature on nucleotide-dependent channel activity (Bijman, Dalemans, Kansen, Keulemans, Verbeek, Hoogeveen, de Jonge, Wilke, Dreyer, Lecocq, Pavirani, and Scholte, 1993).

A scheme for CFTR channel gating that includes phosphorylation and ATP hydrolytic-dependent steps was first proposed by Anderson et al. (1991) and was extended by Haws, Krouse, Xia, Gruenert, and Wine (1992), to include additional channel states. More complex schemes have been proposed which include nucleotide binding at both putative NBDs with hydrolysis occurring at the first NBD (Anderson et al., 1991), the second NBD (Anderson and Welsh, 1992), or both NBDs, but with one hydrolytic event leading to channel opening and the second hydrolytic event leading to channel closure (Baukrowitz, Hwang, Nairn, and Gadsby, 1994; Hwang, Nagel, Nairn, and Gadsby, 1994). We (Venglarik et al., 1994) proposed a scheme to account for details of the gating properties seen at 37°C that is an extension of that proposed by Winter, Sheppard, Carson, and Welsh (1994). The kinetic scheme presented as Scheme I, requires the presence of ATP for channel gating to occur, but neither requires nor precludes ATP hydrolysis as a step in this gating.

CFTR
closed$_0$

$$
\text{PKA (ATP)} \Big\updownarrow \text{PPase}
$$

$$
\underset{K_S = 24\ \mu M}{\overset{\text{ATP}}{\underset{\text{closed}_1}{\text{CFTR}\circledP}} \rightleftharpoons} \underset{6.5\ s^{-1}}{\overset{5.4\ s^{-1}}{\underset{\text{closed}_2}{\text{CFTR}\circledP \text{ATP}}} \rightleftharpoons} \underset{\text{open}}{\text{CFTR}\circledP \text{ATP}} \rightleftharpoons \underset{\text{closed}_3}{\text{CFTR}\circledP \text{ATP}}
$$

SCHEME I

According to this model, quiescent CFTR chloride channels (closed$_0$) are phosphory-lated by PKA in the presence of ATP or ATPγS (Anderson et al., 1991; Tabcharani, Chang, Riordan, and Hanrahan, 1991; Bear, Li, Kartner, Bridges, Jenson, Ramjee-singh, and Riordan, 1992; Cliff, Schoumacher, and Frizzell, 1992; Denning, Ander-son, Amara, Marshall, Smith, and Welsh, 1992; Tilly, Winter, Ostedgaard, O'Riordan, Smith, and Welsh, 1992). PKC may also function at this step in CFTR activation, although, alone it stimulates only ~10% of the current stimulated by PKA under similar conditions (Tabcharani et al., 1991; Berger, Travis, and Welsh, 1993; Chang, Tabcharani, Hou, Jensen, Kartner, Alon, Hanrahan, and Riordan, 1993; Dechecchi, Tamanini, Berton, and Cavrini, 1993). PKA-mediated phosphorylation moves the CFTR channel from an ATP-insensitive, inactive state (closed$_0$) to an ATP-sensitive, activated closed state (closed$_1$). Phosphatases catalyze the reverse reaction to inacti-vate CFTR (Tabcharani et al., 1991; Berger, et al., 1993; Hwang, Nagel, Horie, and Gadsby, 1993; Baukrowitz et al., 1994). The opening of phosphorylated channels from the closed$_1$ to the open state requires the continued presence of ATP, but not PKA (Anderson et al., 1991; Nagel et al., 1992; Venglarik et al., 1994). Questions remain, however, regarding the mechanism of ATP-dependent gating of the phos-phorylated channel. Our previous studies have shown that the open probability (P_o) of stably active CFTR increases as a simple, saturable function of ATP concentration (Venglarik et al., 1994). In these studies P_o reached a maximum value (P_o)$_{max}$ of 0.44 ± 0.04 at maximally effective ATP concentrations. A (P_o)$_{max}$ of ~0.35 to 0.5 has been widely reported (Dalemans, Barbry, Champigny, Jallat, Dott, Dreyer, Crystal, Pavirani, Lecocq, and Lazdunski, 1991; Tabcharani et al., 1991; Anderson and Welsh, 1992; Bear et al., 1992; Denning et al., 1992; Sheppard, Rich, Ostedgaard, Gregory, Smith, and Welsh, 1993; Venglarik et al., 1994) although a variety of P_o have been reported in some conditions (Haws et al., 1992). The gating scheme proposed by Venglarik et al. (1994), and shown as Scheme I accounts for a (P_o)$_{max}$ of less than unity by interposing an ATP-bound-closed state (closed$_2$) between the phosphorylated closed state (closed$_1$) and the ATP-bound open state. The relation of P_o to ATP concentration was described by a single-site interaction between ATP and CFTR. There was no indication from this work (Venglarik et al., 1994) or from a kinetic analysis of CFTR by Winter et al. (1994), for a more complex gating scheme involving two ATP binding sites, as might be anticipated from the domain structure of CFTR, and which has been proposed from functional studies of NBD mutations (Anderson and Welsh, 1992). However, based on studies employing hydrolytic inhibitors which required the concomitant presence of ATP and PKA, Baukrowitz et al. (1994) and Hwang et al. (1994), have proposed a cyclical scheme which includes ATP binding and hydrolysis at two sites to support maximal CFTR channel activity.

The closed$_3$ state in Scheme I was proposed by Haws et al. (1992), although there is previous evidence of its existence (Bear, Duguay, Naismith, Kartner, Hanrahan, and Riordan, 1991; Cliff et al., 1992). The authors (Haws et al., 1992) noted that this short-lived state appeared to differ from the voltage-dependent short-lived state which occurs in cell-attached patch recordings and is currently being studied by other laboratories (Fischer and Machen, 1994). Winter et al. (1994), noted an ATP-independent short-lived closed state, although the kinetic scheme they proposed did not account for an ATP-independent short-lived closed state separate from the transition state between the ATP-sensitive closed state (closed$_1$ of Scheme I) and the

open state; i.e., this proposed scheme did not differentiate between the $closed_2$ and $closed_3$ states presented in Scheme I. The presence and relevance of this short-lived ATP-independent closed state in excised membrane patches was discussed in earlier studies by our laboratory (Venglarik et al., 1994). Our analysis indicates that it is a separate state from $closed_2$, however, we are not able to determine if this state resides between the $closed_2$ and open states or was distal to the open state of the CFTR channel. In any case, the $closed_3$ state accounts for $<3\%$ of the measured P_0 (Venglarik et al., 1994). Therefore, additional studies to determine its location in the kinetic scheme have not been pursued.

Previous reports have indicated that ATP regulates CFTR channel activity over the concentration range of 5 μM to 3 mM; however, cellular ATP concentrations are at the upper limit of this range such that ATP-dependent channel regulation would not likely take place in vivo. If ADP antagonizes the effect of ATP, as suggested by previous studies (Anderson and Welsh, 1992; Winter et al., 1994; Gunderson and Kopito, 1994), then the presence of the nucleotide diphosphate could shift the P_0-ATP curve to the right and perhaps into the physiological ATP concentration range. Therefore, it is important to quantify the inhibitory effect of ADP on CFTR channel activity to come to a better understanding of the physiological control mechanisms for chloride transport and the possible coupling between chloride secretion and cellular metabolic status.

The ATP binding site(s) of CFTR is apparently promiscuous at the nucleotide base site because many nucleotide triphosphates (ATP, GTP, ITP, CTP, UTP, 8-azido-ATP) can support channel activity (Anderson et al., 1991; Travis, Carson, Ries, and Welsh, 1993; Schultz, B. D., unpublished observations). However, compounds having modifications in the polyphosphate moiety (including truncation or substitution, e.g., ADP or AMPPNP), which render these ATP analogues stable to hydrolytic enzymes, do not, by themselves, support CFTR channel gating in excised membrane patches (Anderson et al., 1991; Nagel et al., 1992; Anderson and Welsh, 1992; Carson and Welsh, 1993; Hwang et al., 1994). Based on these observations, it was concluded that ATP hydrolysis is required for CFTR channel gating (Anderson et al., 1991). However, a complete analysis of analogue effects on CFTR channel activity has not been reported. Because the analogues employed in these studies have been shown not to interact with some ATPases (Moos, Alpert, and Myers, 1960; Yount, 1975), it is important to show that the analogues are capable of binding to CFTR and altering nucleotide-dependent function before conclusions can be made regarding a requirement for ATP hydrolysis at either putative nucleotide binding site.

To address these issues, we studied CFTR chloride channel activity in membrane patches excised from CFTR transfected mouse L cells (Yang, Devor, Engelhardt, Ernst, Strong, Collins, Cohn, Frizzell, and Wilson, 1993). The results indicate that ADP is a competitive inhibitor of ATP-dependent CFTR channel gating with an apparent affinity similar to that of ATP. AMP and nonhydrolyzable ATP analogues with alterations in the phosphate chain failed to affect ATP-dependent regulation of CFTR channel gating. This observation precludes any inference regarding the requirement for ATP hydrolysis as a step in CFTR chloride channel activity, but the results do offer insight into the chemical specificity of the interaction of CFTR with ATP.

METHODS

Patch clamp experiments were performed using excised inside-out membrane patches from CFTR transfected mouse L cells (Yang et al., 1993). The data were acquired and analyzed as described previously (Venglarik et al., 1994) with minor modifications. All experiments were performed at 34–37°C unless otherwise stated with membrane potential held at −80 mV (bath vs pipette) so that negative-going currents (represented as downward deflections) represent chloride channel openings. Cells were exposed to forskolin (2–5 μM) to endogenously phosphorylate CFTR before patch excision into a bath which contained 0.3 mM ATP. In 19 of 31 patches used to evaluate the effects of ADP, the catalytic subunit of cyclic AMP-dependent protein kinase (PKA; 150–200 U/0.75 ml; generously supplied by Dr. G. Johnson, University of Alabama at Birmingham [Birmingham, AL], or Promega Corp. [Madison, WI]) was added to the bath after patch excision to insure complete CFTR channel activation and then removed from the bath before data acquisition. Analysis of control period data showed that the method of channel activation (forskolin before patch excision or PKA after patch excision) did not affect subsequent channel activity (data not shown). Channel activity was assessed for a control period of 85–230 s to insure that the CFTR present in the patch was stably active. Unless otherwise noted, the 0.75 ml bath was refreshed at a rate of four bath volumes per minute during the control and treatment periods. Whenever possible, depending on patch viability, a washout (control) period followed the treatment period to document reversibility of effects. Recordings 85–410 s in length were analyzed for each control or experimental condition. Single-channel current (i) was determined based on multi-Gaussian fit of current records. Amplitude histograms were constructed without constraining the peak amplitudes to be equally spaced to further document that an homogenous population of channels was being evaluated. Mean channel amplitude during each control or treatment period was calculated as the average distance between peaks. Mean current (I) was determined by averaging all data points in the current record during the control or treatment period. Current records were visually examined for the duration of patch viability to determine the number of channels (N) present in the patch (i.e., the maximum number of channels simultaneously open in conditions that maximize channel activity (Horn, 1992; Venglarik et al., 1994). Values of i, I, and N were used to calculate the channel open probability (P_o) from the equation

$$P_o = \frac{I}{N * i}.$$ (1)

Only patches containing less than eight channels were evaluated for statistical analysis (Venglarik et al., 1994). Fluctuation analysis and estimation of the corner frequency (f_c), was performed using Bio-Patch software (version 3.11; Molecular Kinetics Inc., Pullman, WA) as previously described (Venglarik et al., 1994). Nonlinear fits of the data for concentration-response relationships were completed using SigmaPlot (versions 4.1 and 5.0, Jandel Scientific, San Rafael, CA). Values are presented as the mean and SEM. Paired t tests were performed using SigmaPlot (version 4.1) to determine significance of treatment effects. When data from a washout period was available, treatment parameters were compared to the average of the parameters recorded during control and washout periods (see Fig. 3). When the patch did not remain viable for a washout period, comparisons were made between treatment and the prior control period. Effects are considered significant if $P < 0.05$ for type I errors.

Single-Channel Recording and Analysis

Of 36 experiments included in this report regarding the effect of ADP, a single experiment was obtained in which only one CFTR chloride channel was present in the excised membrane patch. The analogue recording and digital acquisition apparatus were as previously described.

Digitized files of 230 s (ATP) or 410 s (ATP + ADP) duration were analyzed using Bio-Patch software as previously described with the exception that the PDS was examined to an upper frequency of 140 Hz. Bio-Patch software was employed to determine mean channel state lifetimes and to construct event duration histograms using a half amplitude threshold, a minimum dwell time of 7.5 ms, and a bin width of 10 ms. Event durations (1150 events, ATP; 1170 events; ATP + ADP) were fit by a single (open) or double (closed) exponential decay using Bio-Patch software.

Solutions

The pipette solution contained (in millimolar): 160 N-methyl-D-glucamine-HCl (NMDG-Cl), 1 $CaCl_2$, 2 $MgCl_2$, 10 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (BTP), pH 7.35 ± 0.05. The bathing solution contained (in millimolar): 150 NaCl, 5 KCl, 2 $MgCl_2$, 10 NaF, 0.5 ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.26 $CaCl_2$, and 10 BTP, pH 7.35 ± 0.05. Free Ca^{2+} concentration in the bath was calculated to be 100 nM (Fabiato and Fabiato, 1979). F^- was included as a nonspecific inhibitor of any phosphatases that might be present at excision and can lead to channel inactivation (Tabcharani et al., 1991). Some experiments were performed in the absence of F^- and the outcomes were unchanged. K^+ could also be removed from the bath without any change in channel properties (Schultz, B. D., unpublished observations).

ATP and its analogues were made as stock solutions (200 mM) in 200 mM BTP. The pH of the stock solutions was adjusted to 7.2. ATP was dissolved and aliquots frozen at $-20°C$. All other nucleotide solutions were used within 24 h. Nucleotides were introduced into the bath either by diluting the stock solution into the bath perfusion solution or by pipetting the nucleotide stock directly into the static bath.

Chemical Sources

ATP, AMP, α,β-methyleneadenosine 5'-triphosphate (AMPCPP), β,γ-methyleneadenosine 5'-triphosphate (AMPPCP), and 5'-adenylylimidodiphosphate (AMPPNP) were obtained from Sigma Chemical Co. (St. Louis, MO). ATPγS was purchased from either Calbiochem (La Jolla, CA), or Boehringer Mannheim (Indianapolis, IN). ADP was purchased from Calbiochem. All other chemicals used were reagent grade.

RESULTS

ADP Reduces CFTR Channel Open Probability

Fig. 1 shows two excerpts from a continuous current record with typical CFTR chloride channel gating in the presence of ATP or ATP plus ADP at a holding potential of -80 mV (extracellular reference; downward transitions indicate inward currents; chloride flux from both to pipette). In the presence of 0.3 mM ATP (Fig. 1), CFTR channel activity was similar to that previously reported (Venglarik et al., 1994). The patch contained a maximum of seven simultaneously open channels ($N = 7$). Multi-Gaussian fit of the current record yielded a single-channel amplitude (i) of 1.01 ± 0.01 pA. Mean current (I) during this portion of the record was 4.00 pA. Calculated open probability was 0.57 which is near the upper limit of expected channel activity for these conditions (Venglarik et al., 1994).

The bathing solution was changed to include ADP (0.3 mM) in the continued presence of 0.3 mM ATP (Fig. 1). It can be seen that ADP reduced the maximum number of simultaneously open channels to four, reduced I to 0.96 pA, but did not

alter i (0.99 ± .01 pA). The reduction in mean current could have been caused by either a reduction in the number of available channels (reduction in N), a reduction in P_o, or both. If the inclusion of ADP reduced N from 7 to 4, then P_o was reduced from 0.57 to 0.24; if ADP did not change N from 7, then P_o was reduced to 0.14. Subsequent data will show that P_o was, in fact, reduced without a change in N.

The reduction in CFTR channel activity caused by ADP was overcome by increasing the ATP concentration (Fig. 1). The bathing solution was changed to include 3 mM ATP in the continued presence of 0.3 mM ADP. After ATP addition, six simultaneously open channels were seen. Single-channel amplitude was slightly reduced (0.89 ± 0.01 pA), I was increased to 3.21 pA, and P_o was increased to 0.52 (assuming $N = 7$). Because an increased concentration of ATP could overcome the effect of ADP to inhibit CFTR channel activity, we concluded that ADP did not inactivate channels (reduce N), but, that the effect of ADP was to reduce P_o of activated channels.
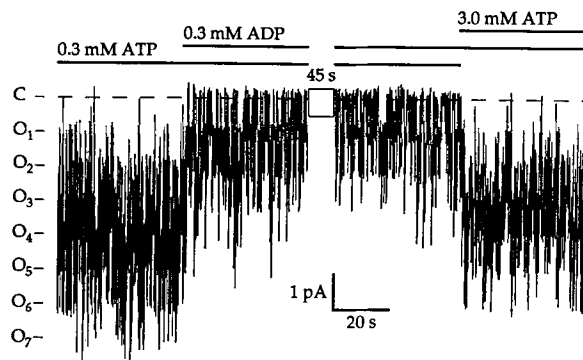

FIGURE 1. Effect of ATP and ADP on CFTR chloride channel open probability. A continuous current record from an excised inside-out membrane patch containing seven CFTR chloride channels is displayed. The patch was held at −80 mV bath relative to pipette such that downward transitions indicate channel opening. The dashed line indicates the current level when all channels were closed and open levels are indicated. Data were filtered at 200 Hz, sampled at 400 Hz, and for presentation, plotted at 100 Hz. During the recording the bath was continually refreshed with a turnover rate of four bath volumes per minute. Bars indicate nucleotide concentrations of the inflowing bath solution. Other conditions were as indicated in the text.

Results shown in Fig. 1 in which ADP reduced ATP-dependent CFTR P_o are typical of 36 such experiments. In each experiment, CFTR channel activity was evaluated for 85 to 230 s in the presence of 0.3 mM ATP. The solution bathing the cytoplasmic face of the membrane was then changed to a combination of ATP (0.3 mM) plus ADP (0.03–3 mM) and channel activity reevaluated. The bathing solution was then returned to the initial conditions and channel activity reassessed, provided that the patch remained viable (13 experiments). Channel open probability in each set of conditions was calculated as previously described (Eq. 1) assuming that N was unchanged during the duration of patch viability. Performed in this manner, each patch served as its own control for the effect of ADP on ATP-dependent CFTR channel activity. Results from these experiments are plotted in Fig. 2. In the presence of 0.3 mM ATP the mean open probability was 0.36 ± 0.01 (mean ± SEM; range = 0.17–0.57). A statistically significant, concentration-dependent reduction in P_o was seen at all ADP concentrations greater than 30 μM. The maximum concentration of ADP used was 3 mM and caused P_o to drop to 0.07 ± 0.02 ($n = 6$). In the

presence of ADP (1–3 mM), but in the absence of ATP, P_o of CFTR chloride channels was 0.02 ± 0.01 ($n = 6$), a value not significantly different from zero.

ADP Does Not Cause CFTR Channel Inactivation

The reduction in CFTR channel P_o could be caused by channel inactivation as previously stated, although the data presented in Fig. 1 would suggest otherwise. To evaluate this possibility, we analyzed data from control and washout periods with intervening ADP exposure. In Fig. 3 A, data from 10 experiments employing three concentrations of ADP are presented. Data from three experiments represented by the open circles show that P_o was 0.38 during control periods. Addition of 3 mM ADP reduced P_o to 0.04 (assuming no change in N). ADP was then removed from the bath, the number of observed channels increased to pretreatment control values and P_o increased to 0.31. Other data presented in Fig. 3 A show that the reduction in P_o associated with lower concentrations of ADP (0.3 and 0.1 mM) is also reversible upon
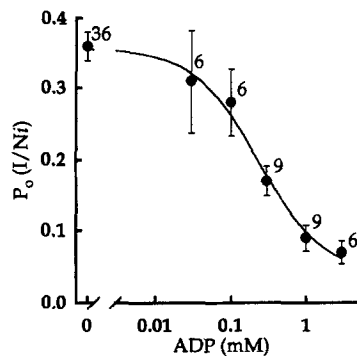

FIGURE 2. Relationship of CFTR chloride channel P_o to ADP concentration. Control records in the presence of 0.3 mM ATP were made from each membrane patch and P_o calculated as indicated for a period >85 s. The bath was then changed to include some concentration of ADP as shown and P_o again calculated such that each patch was used for a control and treatment period to allow for paired statistical analysis. Significant reduction in P_o was seen at all concentrations of ADP tested >30 μM. Data presented are the mean $\pm$ SEM for the number of observations shown at each data point. The solid line indicates the least squares fit of the data to Eq. 2 (see text). $(P_o)_{max}$ and $(P_o)_{min}$ were predicted to be 0.35 ± 0.05 and 0.04 ± 0.04, respectively. K_i (ADP) was estimated to be 16 ± 9 μM.

the removal of ADP. In Fig. 3 B are shown the average P_o from 13 paired control and washout periods. Although a slight reduction in P_o is suggested by these data, this reduction did not approach statistical significance ($P > 0.10$). If channels were inactivated by exposure to ADP or were undergoing time-dependent inactivation (run down), then, the P_o derived by these calculations which employ a constant N would decrease. The data demonstrate that, even at the highest concentration employed, ADP did not inactivate CFTR channels and that time-dependent inactivation did not perceptibly occur. Therefore, we concluded that ADP reduced CFTR channel P_o and that CFTR was stably active for the duration of these experiments.

ADP Is a Competitive Antagonist of ATP-dependent CFTR Channel Opening

The data in Fig. 1 demonstrate that ADP inhibits the effect of ATP on CFTR chloride channel gating and suggest a competitive mechanism of inhibition. To further test

this idea, a series of experiments was performed to determine if the inhibition was competitive, noncompetitive, or uncompetitive. In the case of competitive inhibition, the apparent affinity for ATP would be reduced, but the effect of maximal ATP concentrations on P_o would be unchanged. In the case of either noncompetitive or uncompetitive inhibition the $(P_o)_{max}$ for ATP would be reduced by ADP. To address this question, CFTR channel activity was evaluated in the presence of 5 mM ATP, a concentration more than 100-fold higher than the previously reported K_D for ATP of 44 μM (Venglarik et al., 1994). ADP (0.3 mM) was then introduced into the bath and channel activity again assessed. In five such experiments, P_o was unchanged by the introduction of ADP (5 mM ATP, $P_o = 0.36 \pm 0.04$ vs 5 mM ATP + 0.3 mM ADP, $P_o = 0.37 \pm 0.04$). Results presented in Fig. 2 show that 0.3 mM ADP caused a 50%
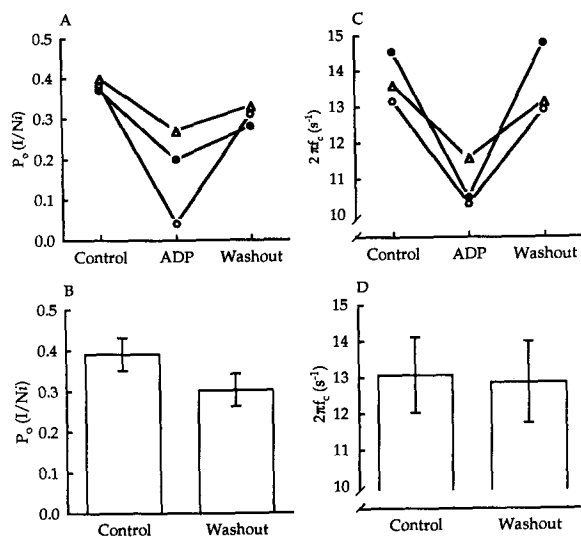

FIGURE 3. Reversibility of ADP-dependent effects on CFTR channel activity. (*A* and *B*) Reversibility of ADP-dependent reduction in P_o. (*A*) Control records in the presence of 0.3 mM ATP were made from each membrane patch and P_o calculated as indicated in the text for a period >85 s. The bath was then changed to include ATP and some concentration of ADP (□ 3 mM, $n = 3$; ● 0.3 mM, $n = 4$; △ 0.1 mM, $n = 3$) as shown and P_o calculated. ADP was removed from the bath and P_o again calculated. (*B*) Mean and SEM of P_o for all experiments in which paired control and washout observations were available ($n = 13$). Paired analysis showed no significant difference between means ($P > 0.10$). (*C* and *D*) Reversibility of ADP-dependent reduction in gating frequency. Experimental conditions and protocols are the same as in *A* and *B*, respectively. Means of control and washout were not significantly different ($P > 0.80$). During data acquisition, the bath was continually refreshed with a turnover rate of four bath volumes per minute containing appropriate nucleotide concentrations.

reduction in P_o (range = 29 to 75%) in the presence of 0.3 mM ATP. ADP inhibition of the effect of submaximal concentrations of ATP while not altering the effect of a maximally effective concentration of ATP is not consistent with either noncompetitive or uncompetitive inhibition. Therefore, we conclude that ADP competitively inhibits ATP-dependent CFTR channel gating.

The data in Fig. 2 were fit to a Michaelis-Menten type equation consistent with competition between ATP and ADP for a single active site (Segel, 1976; discussed in

Appendix) with the following form:

$$P_o = (P_o)_{max} * \left(\frac{\dfrac{[ATP]}{K_s}}{1 + \dfrac{[ATP]}{K_s} + \dfrac{[ADP]}{K_i}} \right) + (P_o)_{min}. \qquad (2)$$

K_s is the Michaelis constant for ATP which was previously determined to be 24 ± 8 µM (Scheme I; Venglarik et al., 1994), and K_i is the inhibition constant for ADP determined by the best fit to the data. ATP concentration was 0.3 mM throughout. The fit of these data yields a $(P_o)_{max}$ of 0.35 ± 0.05 and a K_i for ADP of 16 ± 9 µM. $(P_o)_{min}$ was estimated from this fit to be 0.04 ± 0.04. Zero lies within a 95% confidence interval of $(P_o)_{min}$, and is therefore not significantly different from it. A fit of the data was also made in which $(P_o)_{min}$ was forced to zero. Under this additional constraint, $(P_o)_{max}$ was not changed, but K_i was estimated to be 20 µM. When the constraint of $K_s = 24$ µM was removed, a fit of the data yielded estimates of 30 and 20 µM respectively for K_s (ATP) and K_i (ADP). $(P_o)_{min}$ and $(P_o)_{max}$ were not different from predictions made when K_s was constrained to 24 µM. These results are in close agreement with those reported previously ($K_s = 24 \pm 8$ µM; $P_o = 0.42 \pm 0.03$ at 0.3 mM ATP; Venglarik et al., 1994).

ADP Reduces the CFTR Channel Opening Rate

Data presented in Figs. 1–3 show that ADP competitively reduces ATP-dependent CFTR Cl$^-$ channel P_o. The observations presented, however, do not fully address the mechanism of this inhibition. A simple model of CFTR channel gating has been previously proposed which contains four closed states and a single open state (Scheme I). ADP might interact with any of these states to cause a reduction in P_o. However the data presented in Figs. 1 and 3 suggest that ADP does not inactivate the channel to the closed$_0$ state because inactivation was reversible in the absence of PKA. To gain insight into the mechanism of ADP inhibition of CFTR channel activity, fluctuation analysis was employed to assess possible changes in channel gating resulting from ADP addition.

Using the proposed model of CFTR channel gating (Scheme I), we can predict the effect on channel gating of an ADP interaction with any one of the channel states. The most attractive hypothesis is that ADP interacts with the ATP binding site(s) of the CFTR channel so that the probability of the ATP-occupied closed$_2$ state is reduced. This would be seen in the frequency spectrum as an ADP-dependent decrease in the corner frequency due to a reduction in the channel opening rate (Venglarik et al., 1994) or the addition of a lower frequency Lorentzian component. Alternatively, if ADP interacts with the open state or the short-lived closed state (closed$_3$) of the channel to introduce a closed or blocked state, one would expect an increase in the corner frequency. Each of these possibilities could also be associated with the introduction of an additional Lorentzian component into the power spectrum (i.e., an additional closed or blocked state of the channel).

In Fig. 4 A is shown the power density spectrum from a patch containing two CFTR chloride channels in the presence of 0.3 mM ATP. As was previously demonstrated

(Venglarik et al., 1994), a single Lorentzian component was seen in the frequency range of 0.05 to 30 Hz. For this patch, the corner frequency (f_c) was 2.5 Hz. Introduction of 0.3 mM ADP into the bath caused a decrease of the f_c to 1.7 Hz (Fig. 4 *B*) which corresponds to a reduction in channel gating frequency of 5 s^{-1}.[1] No other Lorentzian components were introduced into the power spectrum by ADP addition (in the range 0.05–140 Hz; although another Lorentzian component was noted both in control and treatment conditions; see Fig. 7). The three low-frequency data points in Fig. 4 *B* might suggest that a very low frequency component has been introduced into the PDS and is skewing the estimate of the more prominent Lorentzian component. To verify that this was not affecting the interpretation of the
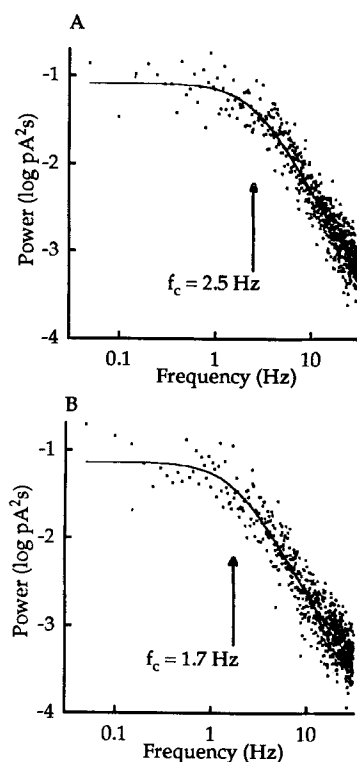

FIGURE 4. Effect of ADP on the power density spectra calculated from the current record of a membrane patch containing two CFTR chloride channels. (*A*) Power density spectra calculated from a current record made in the presence of 0.3 mM ATP. A single Lorentzian component was fit (*solid line*) in the frequency range 0.05 to 30 Hz with a corner frequency (f_c) of 2.5 Hz as indicated. (*B*) Power density spectra calculated from a current record made from the same membrane patch in the presence of 0.3 mM ATP plus 0.3 mM ADP. Introduction of ADP caused a leftward shift of f_c to 1.7 Hz. Data were filtered at 200 Hz and sampled at 400 Hz. No other Lorentzian components were seen in the frequency range of 0.05–140 Hz in either set of conditions. Other conditions were as indicated in the text.

outcome, the same data were analyzed over the frequency range of 0.2 to 30 Hz to eliminate these low-frequency observations. The outcome was unchanged in that the effect of 300 µM ADP was to reduce f_c by 0.55 ± 0.12 Hz when analyzing the frequency range of 0.05 to 30 Hz and by 0.58 ± 0.12 when analyzing the frequency range of 0.2 to 30 Hz (n = 9; P = 0.85). This analysis does not rule out the possibility of a lower frequency Lorentzian component in the power spectrum, but verifies that,

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[1] Most simply, f_c relates to the gating frequency by the equation $2\pi f_c$ = opening rate + closing rate. Therefore, Δf_c multiplied by 2π results in the change in the sum of the opening and closing rates. See Appendix for a more complete discussion of this relationship and its interpretation.

if such a component is being introduced, it is not altering our estimation of the effect of ADP on the more prominent nucleotide-dependent Lorentzian component with a 1 to 3 Hz f_c.

The ADP concentration dependent reduction in f_c for 31 experiments is shown in Fig. 5.[2] To increase precision of the estimates made from the sample set, only the effect of ADP is shown; i.e., the change in $2\pi f_c$ ($\Delta 2\pi f_c$) caused by the introduction of ADP. The mean corner frequency of all patches in the presence of 0.3 mM ATP before the addition of ADP was 2.17 ± 0.01 Hz ($2\pi f_c = 13.6 \pm 0.1$ s^{-1}; $n = 31$). Over the concentration range of 30 μM to 3 mM, ADP reduced the corner frequency in a concentration-dependent manner. Like the effects on P_o, the effects of ADP on f_c were also reversible upon ADP removal and gating characteristics were stable over the duration of the experiments (Fig. 3, C and D). The data in Fig. 5 are fit by an
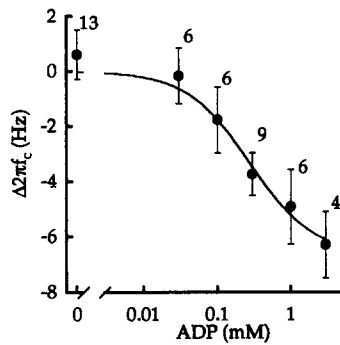

FIGURE 5. Concentration-dependent effects of ADP on CFTR chloride channel gating frequency ($2\pi f_c$). Control records in the presence of 0.3 mM ATP were made from each membrane patch and $2\pi f_c$ calculated. The bath was then changed to include some concentration of ADP as shown and $2\pi f_c$ again calculated and the effect of ADP on $2\pi f_c$ determined ($\Delta 2\pi f_c$). Significant reduction in $2\pi f_c$ was seen at all concentrations of ADP tested > 0.1 mM. Data presented are the mean ± SEM for the number of observations shown at each data point. The solid line indicates the least squares fit of the data to Eq. 3 (see text). In the presence of 0.3 mM ATP $2\pi f_c = 13.6 \pm 0.1$ s^{-1}. K_i (ADP) is estimated to be 35 ± 25 μM.

equation for competitive inhibition with the following form (for derivation see Appendix).

$$\Delta 2\pi f_c = (r_+)_{max} * \frac{[ADP]}{K_i * \left(1 + \dfrac{[ATP]}{K_D}\right) + [ADP]} \tag{3}$$

$(r_+)_{max}$ is the ATP-dependent opening rate in the absence of ADP, K_D was previously determined to be 44 μM (Venglarik et al., 1994), and the ATP concentration was 0.3 mM. The K_i predicted from these data was 35 ± 25 μM; in good agreement with the model independent estimate based on measurements of P_o (16 ± 9 μM). $(r_+)_{max}$ was predicted to be 6.7 ± 1.5 s^{-1} which agrees with our earlier prediction of CFTR opening rate (5.4 ± 0.4 s^{-1}; Venglarik et al., 1994). These results are consistent with ADP interacting with the closed$_2$ state of CFTR shown in Scheme I.

[2] Note that the number of observations at 1 and 3 mM ADP is reduced from those presented in Fig. 2 although this is the same overall data set. In these instances, P_o was sufficiently low while ADP was present such that the PDS did not contain a resolvable f_c in this frequency range.

ADP Increases the Duration of a Long-lived Closed State of the CFTR Channel

Results from a single experiment comparing the activity of a single CFTR chloride channel in the presence of ATP (0.3 mM) and ATP + ADP (0.3 mM) were obtained while completing the 36 experiments included in this report. Excerpts from this record are presented for visual inspection in Fig. 6. In the presence of 0.3 mM ATP, P_o for this channel was 0.46. The power density spectra contained a Lorentzian component with f_c = 2.0 Hz (Fig. 7 A). Because of exceptionally low seal noise in this recording, a second Lorentzian component with f_c = 37 Hz could be resolved (Fig.
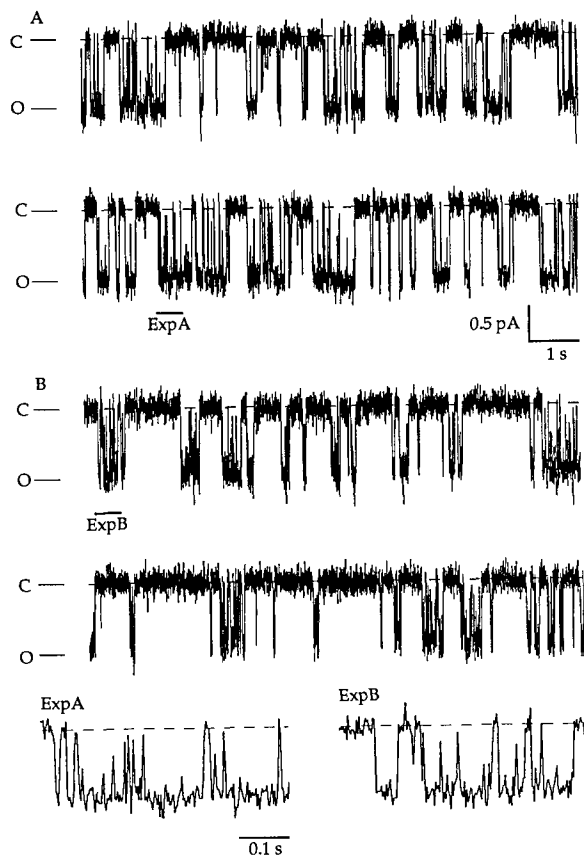

FIGURE 6. Effect of ADP on CFTR channel activity. (A) Channel activity of a single CFTR chloride channel in the presence of 0.3 mM ATP. Data were acquired for a total of 230 s in these conditions. For this portion of the record, i = 1.01 pA, I = 0.45 pA, P_o = 0.45. (B) Gating activity of the same channel presented in A after the addition of 0.3 mM ADP. Data were acquired for a total of 410 s in these conditions. For this portion of the record, i = 1.02 pA, I = 0.24 pA, P_o = 0.24 (ExpA and ExpB) Expanded current traces from A and B as indicated, respectively. Data were filtered at 200 Hz and sampled and plotted at 400 Hz. (Dashed lines) Current level when the channel was closed and open level is indicated. Conditions were as indicated in the text.

7 A). The power associated with this second Lorentzian component was more than two orders of magnitude less than the power of the lower frequency component (6.6 × 10⁻² pA²s vs 5.6 × 10⁻⁴ pA²s). Event duration analysis of this record revealed that open events were characterized by a single exponential decay with τ_o of 91 ms (Fig. 7 B), a value which closely approximates the mean open time of 94 ms. However, closed times were best described by a double exponential decay with τ_{c1} < 7 ms and τ_{c2} = 138 ms (Fig. 7 C). Introduction of 0.3 mM ADP while maintaining ATP at 0.3 mM reduced P_o to 0.23. The power density spectra continued to contain

two Lorentzian components in the range of 0.05 to 140 Hz. The corner of the low frequency Lorentzian component was reduced to 1.7 Hz whereas the f_c of the higher frequency component was not altered (38 Hz; Fig. 7 D). Channel open lifetimes continued to be characterized by a single exponential decay with τ_o of 73 ms (Fig. 7 E). Closed events were fit by a double exponential decay with $\tau_{c1} < 7$ ms and $\tau_{c2} = 261$ ms (Fig. 7 F). The most plausible interpretation of these data is that the introduction of ADP caused a 60% increase in the duration of long-lived closed events (τ_{c2}) while having little effect on τ_o and no perceptible effect on τ_{c1}. An alternative
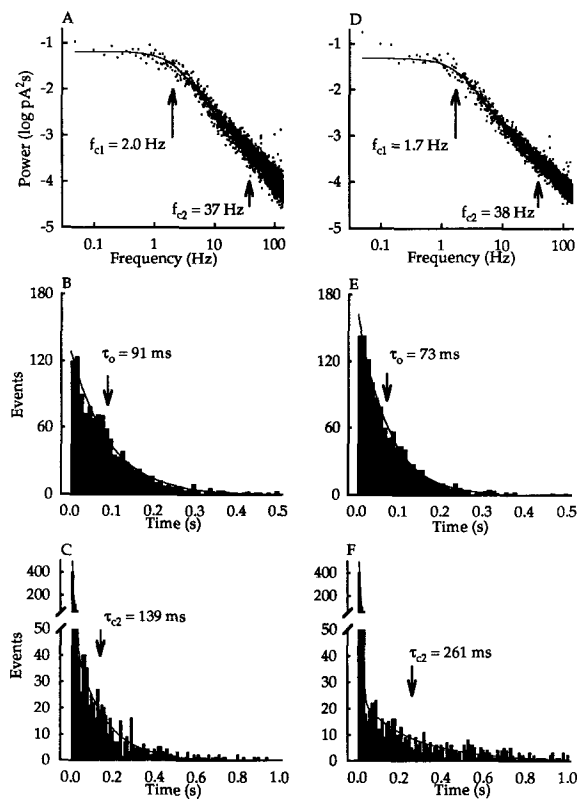

FIGURE 7. Analysis of gating activity of a single CFTR chloride channel as shown in Fig. 6. (A and D) Power density spectra calculated from current records made in the presence of 0.3 mM ATP (A) or 0.3 mM ATP + 0.3 mM ADP (D). Two Lorentzian components were fit ($solid$ $line$) in the frequency range 0.1–140 Hz with corner frequencies (f_c) of 2.0 and 37 Hz (A) or 1.7 and 38 Hz (D) as indicated. (B and E) Channel open duration histograms determined for the same current records described in A and D, respectively. Open durations were fit by a single exponential decay with τ_o as indicated. (C and F) Channel closed duration histograms determined for the same current record described in A and D. Closed durations were fit by a double exponential decay with τ_{c2} as indicated. τ_{c1} was <7 ms in both conditions and was not perceptibly altered by exposure to ADP. Events <7.5 ms were excluded from analysis. For analysis, data were filtered at 200 and sampled at 400 Hz. Conditions were as indicated in the text.

interpretation is that ADP introduced a longer-lived closed state of CFTR, but that the record did not contain sufficient number of events to adequately separate it from τ_{c2}. It should be noted that with 1150 or 1170 events per condition and the limitations of the recording system, τ_{c1} is obviously present, but its duration cannot be predicted with a high degree of accuracy. Additionally, the higher frequency Lorentzian component was obviously present, but contained such low power that both its plateau power and f_c are difficult to determine with reasonable precision.

These results are consistent with Scheme I and the observations reported by Haws et al. (1992).

AMP Does Not Alter CFTR Channel Activity

Similar to ADP, AMP (1 mM) added alone did not support significant levels of CFTR channel activity (Fig. 8 A and Table I). As shown in Fig. 8 A, channel events were
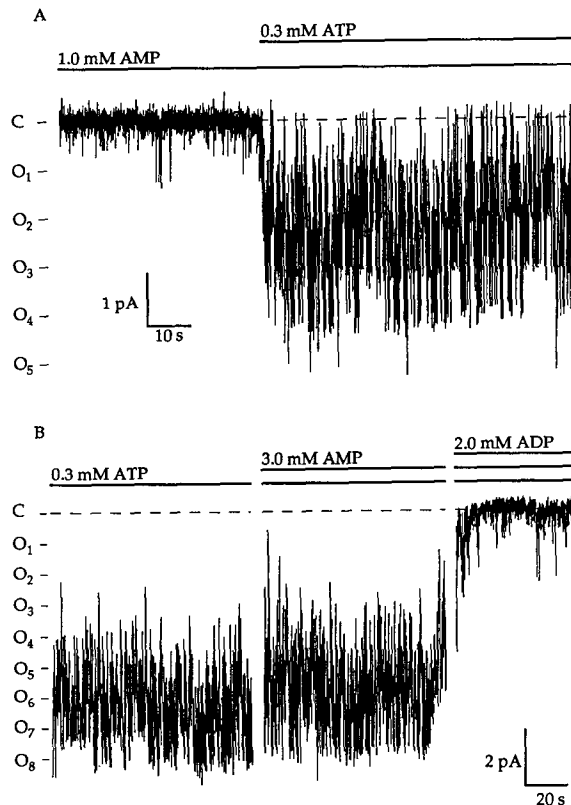

FIGURE 8. Effects of adenine nucleotides, alone and in combination, on CFTR chloride channel open probability (P_o). (A) Typical current record from a membrane patch containing five CFTR chloride channels. In the presence of 1 mM AMP channel events were seldom seen $(P_o < 0.01)$. ATP (0.3 mM) was then perfused into the bath in combination with 1 mM AMP and P_o increased to 0.48. These results are representative from a group of ten such experiments. (B) Typical current record from a membrane patch containing more than eight CFTR chloride channels. Control activity in the presence of 0.3 mM ATP is shown. Introduction of 3 mM AMP into the bath in the continued presence of 0.3 mM ATP did not detectably alter channel activity. Subsequently, the introduction of 2 mM ADP into the bath reduced CFTR P_o. These results are representative from a group of seven such experiments. The dashed lines indicate the current level when all channels were closed and open levels are as indicated at the left. For analysis, data were filtered at 200 Hz and sampled at 400 Hz, however, for presentation, data were plotted at 100 Hz. During the recording for A, the bath was continually refreshed with a turnover rate of four bath volumes per minute. Bars indicate nucleotide concentrations of the inflowing bath solution. During the recording for B, the bath was static and stock solutions of nucleotides were pipetted into the bath. Breaks in the record represent nucleotide addition and mixing. Other conditions were as indicated in the text.

seldom seen in the presence of 1 mM AMP. Subsequently, ATP (0.3 mM) along with AMP was perfused into the bath and channel activity was seen; in this experiment P_o increased from <0.01 to 0.48. 10 such experiments were performed with P_o increasing from 0.02 ± 0.01 to 0.36 ± 0.06 (range = 0.13 to 0.69) in the presence of

ATP. This protocol verifies that 1 mM AMP fails to support CFTR channel activity, but does not indicate if AMP alters the effect of the subsequently added ATP. Therefore, a second set of experiments was completed in which 0.3 mM ATP was present in the bath before and after the addition of 1–3 mM AMP (stock solution pipetted into the bath). In the presence of 0.3 mM ATP, P_o was 0.40 ± 0.05 and was unchanged by the subsequent addition of 1–3 mM AMP (0.40 ± 0.07; $n = 7$; Fig. 8 B and Table I). Furthermore, ADP was subsequently added to the bath and channel inhibition was seen (Fig. 8 B); this verifies that the channels were available for inhibition. In sum, data presented in Fig. 8 indicate that AMP (1 mM) fails to interact with CFTR in a manner that causes any alteration in ATP-dependent channel gating.

Nonhydrolyzable ATP Analogues Do Not Alter CFTR Channel Activity

In an effort to determine if ATP hydrolysis is a requirement for CFTR channel gating, analogues of ATP with an imido- or a methylene linkage between the β and γ-phosphates (AMPPNP, AMPPCP, respectively) were added in the presence and

TABLE I

Open Probability of CFTR Cl⁻ Channels in the Presence of ATP Analogues Alone and in Combination with ATP*

Analogue	Analogue alone	n	ATP plus analogue	n
ATP	0.36 ± 0.01	36	0.36 ± 0.01	36
ADP	0.02 ± 0.01	6	0.09 ± 0.02[‡]	9
AMP	0.02 ± 0.01	10	0.40 ± 0.07[§]	7
AMPPNP	0.05 ± 0.03	4	0.44 ± 0.08[§]	3
AMPPCP	0.01 ± 0.01	4	0.48 ± 0.05[§]	3
ATPγS	0.04 ± 0.00	3	0.38 ± 0.04[§]	4
AMPCPP	0.03 ± 0.01	8	0.33 ± 0.07[§]	6

*All experiments were done on a paired basis with each patch serving as is own control for the effect of nucleotides on CFTR P_o. Therefore, for statistical analysis, comparisons were made between treatments and their paired ATP controls. Data reported are mean and SEM for the number of observations shown. ATP concentration was 0.3 mM. Analogue concentration was 1 mM or greater.

[‡]P_o significantly less ($P < 0.05$) than in the presence of ATP alone.

[§]P_o not significantly different ($P > 0.05$) than in the presence of ATP alone.

absence of ATP. In other systems, replacement of the γ-ester linkage results in compounds that cannot be hydrolyzed by ATPases and, thus fail to support reactions which require ATP hydrolysis as an energy source (Yount, Babcock, Ballantyne, and Ojala, 1971a; Yount, 1975). Furthermore, these substitutions result in compounds which can competitively inhibit hydrolytic reactions (Yount, Ojala, and Babcock, 1971b). Thio- substitution of a nonbridge oxygen on the γ-phosphate (ATPγS) results in a compound which can be used in some kinase catalyzed reactions, but not in ATPase reactions (Eckstein, 1985). Experimental protocols identical to those used to evaluate the effects of AMP and ADP as shown in Fig. 8, A and B, were used. Results from these experiments are summarized in Table I. One of two possible outcomes is expected from these studies. Either the analogues interact with CFTR and fail to support channel transitions (i.e., maintain either an open or a closed state), as did ADP, or the analogues do not interact with CFTR, similar to AMP. If the analogues

interact with the ATP activation site(s) on CFTR, but fail to support channel activity one would expect them to inhibit ATP-dependent channel gating; either to prolong an open state of CFTR as has been reported in some situations, or to prolong a closed state similar to the action of ADP. In the left column of Table I are data which show that neither AMPPNP, AMPPCP nor ATPγS initiated CFTR channel activity when present in the bath at 1 mM. Some low level of channel activity ($P_o \leq 0.05$) was seen in the presence of 1 mM of these three ATP analogues, however, this P_o was not significantly different than zero and could result from ATP contamination of the compounds (Carson and Welsh, 1993; Sigma Technical Assistance, St. Louis, MO). In every case, the subsequent addition of 0.3 mM ATP (a threefold lower concentration than that of the ATP analogue) resulted in a significant increase in P_o (AMPPNP, 0.47 ± 0.03, $n = 4$; AMPPCP 0.36 ± 0.06, $n = 4$; ATPγS, 0.25 ± 0.04, $n = 3$). While there is wide variation in the P_o subsequent to ATP addition in the presence of ATP analogues, the data clearly demonstrate that a threefold lower concentration of ATP is able to stimulate and support a 6- to 12-fold increase in channel P_o compared to the channel activity that was seen in the presence of the ATP analogue alone. This is a clear divergence from other systems in which ATP analogues have been shown to be effective on an equimolar basis at mimicking the effects of ATP (Morley and Stadtman, 1970; Yount, et al., 1971*b*; Hayden, Miller, Brauweiler, and Bamburg, 1993). Subsequent experiments were designed to determine if ATP analogues altered the effect of ATP on CFTR chloride channels.

Results presented in the right column of Table I show that none of these three analogues (AMPPNP, AMPPCP, ATPγS), when used in threefold excess of ATP, altered the ATP-dependent P_o of CFTR. In experiments employing each of these analogues in the presence of ATP, subsequent addition of ADP resulted in a substantial reduction in P_o similar to that seen in Fig. 8 *B*. Because neither channel activation nor inhibition were seen in response to the addition of these nonhydrolyzable ATP analogues, we conclude they fail to interact with CFTR in a manner that alters ATP-dependent channel gating.

A final ATP analogue tested was AMPCPP in which the β-ester linkage is replaced by a methylene linkage. AMPCPP supports ATPase activity in some systems (Yount, 1975; McNaught, Raymoure, and Smith, 1986; Raymoure, McNaught, Greene, and Smith, 1986) and has previously been reported to support CFTR channel activity (Anderson et al., 1991). In contrast to the previous report, AMPCPP failed to support CFTR channel activity and did not inhibit ATP-dependent channel activity (Table I). The reason for the disparity with the results of Anderson et al. (1991) remains unexplained (see Discussion). Because none of the ATP analogues tested effectively interacted with CFTR, no inference regarding ATP hydrolysis as a requisite step in CFTR chloride channel gating can be made based on these results.

PKA and Temperature-dependent Changes in CFTR Channel Activity

AMPPNP and other ATP analogues have been shown to alter CFTR channel activity in some systems. Most notably, Hwang et al. (1994) and Gunderson and Kopito (1994) have shown that AMPPNP causes CFTR to "lock" open. However, to demonstrate this effect, the authors indicated that a PKA dependent, high P_o state was a prerequisite to entering this locked state. When CFTR was not in this putatively

high phosphorylation state, AMPPNP had no effect on channel gating (Hwang et al., 1994).

A series of experiments was completed to evaluate the effects of AMPPNP in the presence of PKA and 0.3 mM ATP. CFTR channel P_0 appeared to slightly increase in response to the continued presence of PKA on the cytoplasmic face of the membrane, but gating characteristics were not perceptibly altered ($P_0 = 0.61 \pm 0.05$; $f_c = 2.80 \pm 0.41$; $n = 6$; e.g., see Fig. 9 A). Paired experiments with appropriate statistical analysis were not performed to determine if the increased P_0 in the presence of PKA was significant. That the P_0 in the presence of PKA was elevated compared to previous observations in the absence of PKA might suggest that CFTR did, in fact, enter an alternative phosphorylation state capable of achieving a higher P_0. AMPPNP was then pipetted into the bath (final concentration of 1 mM). In this
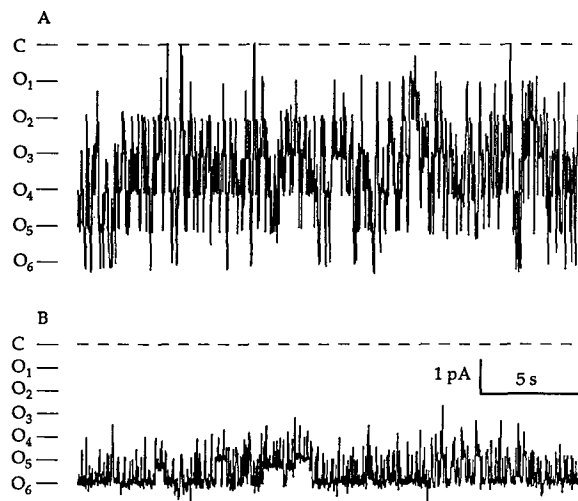

FIGURE 9. Effects of temperature on CFTR chloride channel activity. (A) Current record from a membrane patch containing six CFTR chloride channels at 35°C and in the presence of 0.3 mM ATP and 200 u PKA. $i = 1.10$ pA, $I = 3.87$ pA, $P_0 = 0.59$. (B) Continuation of current record shown in A; temperature = 25°C. Because of cooling, i is decreased when compared to A. $i = 0.70$ pA, $I = 3.90$ pA, $P_0 = 0.93$. Total duration from the start of A to the end of B was 7 min. These results are representative from a group of four such experiments. The dashed lines indicate the current level when all channels were closed and open levels are as indicated at the left. For analysis, data were filtered at 200 Hz, sampled at 400 Hz, however, for presentation data were plotted at 100 Hz. During the recording the bath was static. Other conditions were as indicated in the text.

set of experimental conditions, neither P_0 nor f_c were altered by the addition of AMPPNP ($P_0 = 0.64 \pm 0.05$, $P = 0.10$; $f_c = 2.46 \pm 0.52$, $P = 0.33$; $n = 6$).

Temperature is a second variable that might affect CFTR channel activity and the effects of nucleotides on this activity. Bijman et al. (1993) reported that CFTR remained active in the absence of nucleotides when assayed at 25, but not at 37°C. Devor, Forrest, Suggs, and Frizzell (1994) reported nucleotide-independent channel gating at room temperature and the observations by Hwang et al. (1994) and Gunderson and Kopito (1994), concerning the effect of AMPPNP CFTR channel activity were made at 25°C.

In Fig. 9 A is shown a current record from a patch containing six CFTR channels in conditions similar to those used throughout this report with the exception that PKA was present. Visual inspection suggests that there is little effect of PKA on channel

gating (compare with Figs. 1 and 8). In these conditions, i was 1.10 pA and I was 3.87 pA resulting in a P_o of 0.59.

The thermostat of the Peltier temperature controller was then reduced to cool the bath and patch to 25°C over a 5-min period (we achieved no success in attempts to obtain membrane patches at 25°C). Two effects of reduced temperature on channel characteristics were seen. First, P_o increased dramatically, and second, single-channel amplitude (i) decreased as temperature decreased. As demonstrated in Fig. 9 B, cooling to 25°C resulted in a diminution in i to 0.70 pA and an increase in P_o to 0.93.
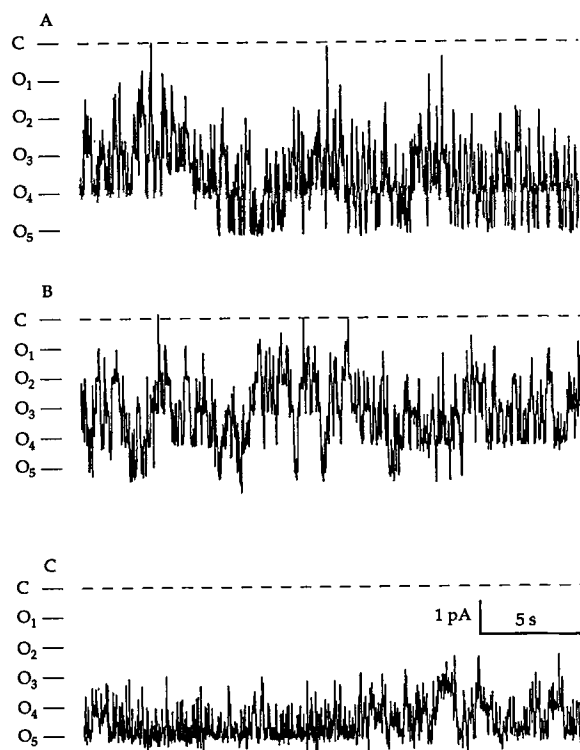

FIGURE 10. Effects of temperature and PKA on CFTR chloride channel activity. (A) Current record from a membrane patch containing five CFTR chloride channels at 35°C and in the presence of 0.3 mM ATP. $i = 1.15$ pA, $I = 4.04$ pA, $P_o = 0.70$. (B) Continuation of current record shown in A; ~4 min later. Temperature = 25°C. $i = 0.93$ pA, $I = 2.99$ pA, $P_o = 0.64$. (C) Continuation of current record shown in A and B; ~20 s after B during which time PKA was pipetted into the bath. Temperature = 25°C. $i = 0.91$ pA, $I = 4.21$ pA, $P_o = 0.93$. Results presented in A and B are representative from a group of four such experiments. The dashed lines indicate the current level when all channels were closed and open levels are as indicated at the left. For analysis, data were filtered at 300 Hz, sampled at 800 Hz, however, for presentation data were plotted at 100 Hz. During the recording the bath was static. Other conditions were as indicated in the text.

These results are typical from five such experiments where P_o increased on average to 0.88 ± 0.04. However, in three such experiments, P_o did not increase with a reduction in temperature.

Similar experiments were performed in the absence of PKA. Results from one such experiment are displayed in Fig. 10. Similar to the previous experiments, cooling the bath to 25°C resulted in a 22 ± 1% reduction in i ($n = 7$). However, only a modest effect on P_o was seen (compare Fig. 10, A with B). Five patches contained few enough channels for complete analysis. In these patches, the reduction in temperature was

accompanied by reduction in P_o from 0.43 ± 0.07 to 0.35 ± 0.09. Two additional patches contained >11 channels and cooling did not cause a perceptible change in gating. Data presented in Fig. 10 A are from a patch containing five channels with a higher P_o (0.70) than was normally seen in these conditions. This unusually high P_o might be indicative of a 'highly phosphorylated state' similar to that seen in the presence of PKA (Hwang et al., 1994), and, as such, might be expected to parallel the results presented in Fig. 9 when PKA was present, i.e., increased P_o with a reduction in temperature. However, P_o was little changed, and actually decreased slightly from 0.70 to 0.64 when temperature was lowered to 25°C. Moreover, when PKA was added to the bath, P_o increased dramatically to 0.93 (Fig. 10 C).

Results presented in Figs. 9 and 10 demonstrate that CFTR is regulated differently at room temperature or 25°C than at 34 to 37°C. Additionally, at least a portion of the alternative gating characteristics is dependent on the presence of PKA. Because CFTR enters a previously uncharacterized, high P_o state simply as a result of reduction in temperature, and this effect appeared to depend on the presence of PKA and ATP, we did not attempt to evaluate further effects of other nucleotides on CFTR channel activity at the lower temperature.

DISCUSSION

This study has three major findings. First, ADP is a competitive inhibitor of ATP-dependent CFTR chloride channel activity with an apparent affinity for the ATP binding site(s) that is similar to that of ATP. Previously, we reported that ATP increases CFTR P_o by increasing the opening rate of the channel (Venglarik et al., 1994). Here we show that ADP decreases CFTR P_o by decreasing the channel's opening rate in a concentration dependent manner. The second major finding is that AMP and the ATP analogues AMPPCP, AMPPNP, ATPγS, and AMPCPP failed to effectively interact with a stably active, previously characterized form of CFTR, either to support channel gating or to inhibit ATP-dependent channel gating. Apparently, the structure of the polyphosphate moiety of these analogues is sufficiently different from ATP that they cannot interact with the ATP binding site(s) on CFTR. Thus, constraints on the interaction of the ATP binding site(s) with nucleotide phosphates are suggested by these findings because differences in the structure of the polyphosphate moieties of these ATP analogues have previously been reported (Yount, 1975). The third finding is that the PKA-dependent regulation of CFTR is temperature sensitive. There have been disparate reports regarding the effects of ATP analogues (Quinton and Reddy, 1992; Bell and Quinton, 1993; Carson and Welsh, 1993; Hwang et al., 1994; Gunderson and Kopito, 1994) and the concentration-dependent effects of ATP on CFTR channel activity (Anderson et al., 1991; Quinton and Reddy, 1992; Bell and Quinton, 1993; Bijman et al., 1993; Venglarik et al., 1994; Devor et al., 1994; Gunderson and Kopito, 1994). The observations which we present regarding the interaction between temperature and PKA-dependent channel activity begin to bridge the gap between these seemingly contradictory reports.

ADP Inhibition of ATP-dependent CFTR Channel Gating

We have shown that ADP does not support CFTR channel activity and that ADP reduces channel activity stimulated by ATP. In other studies (Venglarik et al., 1994),

we found that ATP stimulates CFTR channel activity in a concentration-dependent manner. The effects of ATP on CFTR gating and P_o gave rise to Scheme I (Venglarik et al., 1994) which was an extension of earlier linear kinetic models (Anderson et al., 1991; Haws et al., 1992; Winter et al., 1994). The present work has further verified this model by supporting its estimates of the closing rate and ATP-dependent opening rate of CFTR. Furthermore, it was shown that ADP interacts with the phosphorylated, nucleotide unoccupied form of CFTR to prevent ATP from binding and stimulating channel opening. The location of this interaction is exclusive with the ATP binding site(s) because the ADP effect on P_o is fit well by a model for competition with ATP binding. Alternative mechanisms of interaction were ruled out based on experiments which examined the effect of ADP on maximally stimulated patches (5 mM ATP). Results showed that there was no effect of ADP in the presence of 5 mM ATP ruling out the possibility of either noncompetitive or uncompetitive interactions. If the apparent K_s for ATP is higher than 24 μM as has been reported by other laboratories (Anderson et al., 1991; Quinton and Reddy, 1992; Bell and Quinton, 1993), then the effect of ADP should have been more pronounced at this concentration of ATP.
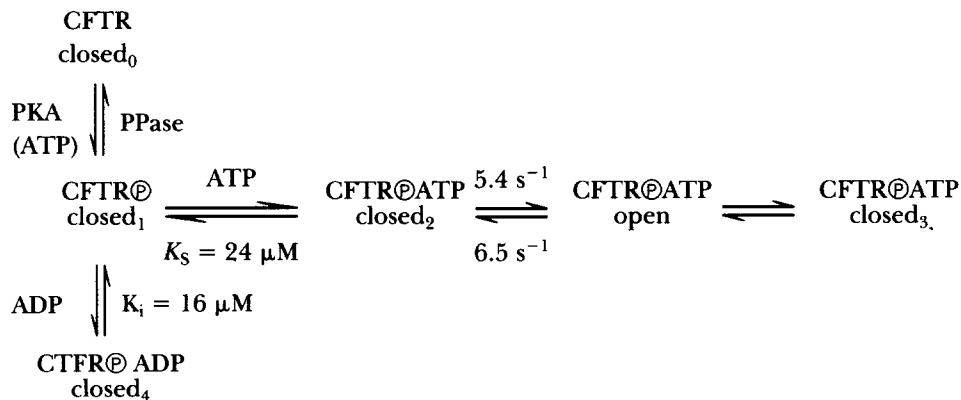
We previously showed that the power spectra associated with CFTR was dominated by a single Lorentzian component in the frequency range of 0.1–30 Hz and that the corner of this component was sensitive to nucleotide concentration (Venglarik et al., 1994). Based on these findings and the earlier report by Haws et al. (1992), kinetic Scheme I was proposed (Venglarik et al., 1994). In full, this scheme might be expected to be associated with up to four Lorentzian components. However, in the absence of PKA, no Lorentzian component would be expected for transitions from the $closed_0$ state. Furthermore, constraining the upper frequency limit to 30 Hz would preclude predicting the frequency of short-lived events ($closed_3$; see Appendix).

Fluctuation analysis indicated that the nucleotide dependent f_c decreased in parallel with P_o. This reduction is consistent with ADP causing a reduction in the ATP-dependent opening rate (prolonging a closed time) of the CFTR channel and supports predictions made by Winter et al. (1994) concerning the effects of ADP. The maximal change in gating ($\Delta 2\pi f_c$; Fig. 5) caused by ADP was to reduce the gating frequency by 6.7 ± 1.5 s^{-1}. The maximal change in $2\pi f_c$ corresponds to the maximal ATP-dependent opening rate of CFTR channels in these conditions and is in reasonable agreement with the previously reported ATP-dependent opening rate of 5.4 ± 0.4 s^{-1} (Venglarik et al., 1994). These results, along with the observation that channel amplitude did not change and that the power spectra in the frequency range of 0.05–140 Hz contained no additional Lorentzian components resulting from the addition of ADP, rule out the possibility that ADP interacts with the open state of CFTR to cause closure. That the channel is reversibly regulated by ATP and ADP in the absence of PKA (Figs. 1 and 3) indicates that ADP does not cause channel inactivation to the $closed_0$ state (see Scheme I). Competitive inhibition of CFTR gating by ADP rules out the possibility that ADP interacts with the $closed_2$ (ATP bound) state.

Interpretation of data from multichannel patches is reinforced by data from one patch containing a single CFTR chloride channel. Similar to multichannel patches,

fluctuation analysis of this single-channel record showed that a single Lorentzian component was present in the frequency range of 0.05–30 Hz and that this f_c was modulated by ADP. When expanded to an upper frequency limit of 140 Hz, a second Lorentzian component not affected by ADP was resolved, but with very low power. ADP did not alter the power or f_c associated with this higher frequency Lorentzian component ruling out the possibility that ADP increases the transition rate to this short-lived closed state (closed$_3$). Like the report by Winter et al. (1994), the current record contained a single population of open times both in the absence and presence of ADP, and this open time was unaffected by ADP addition. Data from this experiment offer additional information not gleaned from the analysis of multi-channel current records by demonstrating a short-lived closed state by both event duration analysis and fluctuation analysis. The duration and frequency of entering this short-lived state was independent of ADP addition and is consistent with the closed$_3$ state of Scheme I as has been previously been reported and discussed (Haws et al., 1992; Venglarik et al., 1994; Winter et al., 1994). Rather, the effect of ADP was to extend the longer-lived closed time of CFTR which has previously been reported (Winter et al., 1994).

All data presented from both multichannel and single-channel patches are consistent with ADP interacting with the closed$_1$ state of CFTR shown in Scheme I in which the interaction of phosphorylated CFTR with ADP precludes ATP binding with the nucleotide binding site(s). Because ADP itself does not permit CFTR to open, its competition with ATP at the nucleotide binding site(s) inhibits CFTR gating. Thus, f_c decreases and the duration of the long-lived closed state seen in a single-channel patch increases. Additionally, the data presented further support the kinetic scheme proposed by Venglarik et al. (1994). Both the maximal gating frequency ($2\pi f_c = 13.6 \pm 0.1$ s^{-1}) and the maximal opening rate (6.7 ± 1.5 s^{-1}) of CFTR channels are in agreement with values previously published (Venglarik et al., 1994). Thus, Scheme II represents a modification of the previous scheme to include this new ADP-bound closed state:

CFTR
closed$_0$

PKA ⇅ PPase
(ATP)

CFTR℗   ⇌ ATP   CFTR℗ATP $\xrightarrow{5.4\ s^{-1}}$ CFTR℗ATP   ⇌   CFTR℗ATP
closed$_1$ closed$_2$ $\xleftarrow{6.5\ s^{-1}}$ open   closed$_3$.

$K_S = 24\ \mu M$

ADP ⇅ $K_i = 16\ \mu M$

CTFR℗ ADP
closed$_4$

SCHEME II

Based on the proposed model for ATP activation of CFTR (Scheme II), an inhibitory constant for ADP of $16 \pm 9\ \mu M$ could be estimated from the current data.

Others (Anderson and Welsh, 1992; Gunderson and Kopito, 1994) previously reported that ADP competitively inhibited CFTR channel activity but defined neither the channel state that was affected by ADP nor the inhibitory constant. Subsequently, Winter et al. (1994) reported that ADP interacted with the ATP-free form of CFTR to preclude ATP binding (i.e., competitive), but did not quantify the inhibition. Quinton and Reddy (1992) reported that, in human sweat duct, ADP stimulated CFTR. However, the authors suggested that this could be the result of phosphorylation of ADP to ATP by endogenous adenylate kinase. Therefore, in the sweat duct, any direct effect of ADP on CFTR may be obscured by uncertainties of local nucleotide concentrations and enzyme activities in these semi-intact permeabilized epithelial cells.

Our results imply that CFTR is capable of sensing cellular energy levels and adjusting channel activity accordingly as previously suggested by Quinton and co-workers (Quinton and Reddy, 1992; Bell and Quinton, 1993). The literature contains many and varied reports of the concentration, either total or free, of adenine nucleotides with ADP concentrations ranging from 0.04 to 2 mM, ATP concentrations ranging from 1.2 to 8 mM, and ATP:ADP ratios ranging from 1.5 to 20 (Veech, Lawson, Cornell, and Krebs, 1979; Dunne, West-Jordan, Abraham, Edwards, and Petersen, 1988; Gembal, Detimary, Gilon, Gao, and Henquin, 1991; Ghosh, Ronner, Cheong, Khalid, and Matschinsky, 1991; Ohta, Nelson, Nelson, Meglasson, and Erecinska, 1991; Owen, and Halestrap, 1993; for review of adenine nucleotide turnover see Hashimoto, Yoshida, and Tagawa, 1990; Sahlin, 1992). Although the predicted K_s (ATP) and K_i (ADP) are substantially lower than total concentrations of cellular ATP and ADP in many of these reports, the relative affinities are such that regulation can occur at the reported physiologic concentrations of these nucleotides. Employing Eq. 2 and the cellular concentrations of ATP (5.75 mM) and ADP (2.03 mM) reported by Dunne et al. (1988) with the affinities we determined, steady state P_o would be 0.24.[3] A conversion of 10% of cellular ATP to ADP would result in a reduction in CFTR P_o to 0.21; a > 10% reduction in P_o. Similar results can be obtained by employing other reported values for ATP and ADP concentrations and performing identical calculations. Thus, regardless of the predicted adenine nucleotide concentrations within the cell, as cellular energy charge falls, and perhaps more importantly the ATP:ADP ratio at the apical membrane falls, the rate of electrolyte transport is reduced by a graded reduction in chloride conductance.

ATP Analogues and the ATP Binding Site of CFTR

Modifications in portions of the molecular structure of a ligand are frequently employed to probe and characterize a binding site. Unlike other modified ligands, nucleotide analogues, if they interact with an acceptor, offer additional information regarding the reaction sequence if a hydrolytic event is required in a reaction scheme (Eckstein, 1985). Analogues which are resistant to hydrolysis include those in which a bridging oxygen in the phosphate chain is replaced by either a methylene- or an

[3] If [ATP] = 5.75 mM, [ADP] = 2.03 mM, K_s (ATP) = 0.024 mM, K_i (ADP) = 0.017 mM, and $(P_o)_{max}$ = 0.37, then, employing Eq. 2, P_o = 0.24.

imido-linkage (Yount et al., 1971a, b; Yount, 1975). Thio-substitution of a nonbridge oxygen on the γ-phosphate also results in an ATP analogue that is useful for kinases, but is resistant to hydrolysis by ATPases (Eckstein, 1985). Our results from experiments employing ATP analogues are in agreement with earlier reports which found that nonhydrolyzable ATP analogues do not support CFTR channel activity when assayed using the membrane patch voltage clamp technique (Anderson and Welsh, 1992; Nagel et al., 1992; Carson and Welsh, 1993) or the planar lipid bilayer technique (Gunderson and Kopito, 1994). In our hands, none of these analogues (AMPPCP, AMPPNP, ATPγS) were able to support CFTR channel activity. The current work extends this observation by showing that these nonhydrolyzable ATP analogues do not interfere with ATP-dependent channel activity. Thus we conclude that these analogues do not interact with this previously characterized, stably active form of CFTR. Additionally, we showed that AMPPNP did not appreciably alter channel gating even when PKA was present to induce a putatively high phosphorylation state of CFTR.

Yount (1975) reported that ester-linkage substitutions result in slight, but significant changes in the structure of the phosphate chain when examined as the tetra-sodium pyrophosphate form (Note that this structural analysis was performed in the absence of nucleosides and divalent cations.). Imido-substitution results in a 3° decrease in the bond angle (P-N-P versus P-O-P) and 8 pm increase in the interphosphate distance while a methylene-linkage (P-C-P) results in a more dramatic 13° reduction in bond angle and 13 pm increase in the interphosphate distance. The effect of such substitutions on molecular structure of a nucleotide triphosphate in the presence of divalent cations is unknown. These analogues, along with ATPγS, also differ from ATP in their ionization constants and in their affinity for divalent metals (Moos et al., 1960; Yount et al., 1971a; Yount, 1975; Eckstein, 1985). However, in the presence of excess Mg^{2+} and at pH 7.35 (the conditions currently used), ATPγS, AMPPNP and AMPPCP would all be expected to be largely in a Mg-bound form (Moos et al., 1960; Yount, 1975), as would >95% of ATP in these conditions (Fabiato and Fabiato, 1979). Therefore, our results suggest that the ATP activation site of CFTR is exquisitely sensitive to alterations in the phosphate chain because the phosphate chain substitutions present in these analogues are likely to increase the affinity for divalent cations and cause little change in ionization at pH 7.35. Mg-ATP can exist in at least four bidentate forms and can rapidly isomerize between these forms in solution while substituted analogues do not (Dunaway-Mariano and Cleland, 1980). Therefore, the lack of activity in the presence of ATP analogues probably results from the formation of stable isomers which contain unfavorable structures for binding in either the chirality of the phosphate chain or in the screw sense (Δ or Λ) of the nucleotide (Dunaway-Mariano and Cleland, 1980; Eckstein, 1985). Further investigation using the stable Cr- or $Co(NH_3)_4$- forms of ATP may overcome the ambiguity that accompanies the use and ineffectiveness of nonhydrolyzable ATP analogues since, in these forms, both the nucleoside and the phosphate chain are unaltered.

The lack of effect of nonhydrolyzable ATP analogues on CFTR might also be explained by the hypothesis that CFTR may be regulated by ATP^{4-}. In this case, the higher affinity of the analogues for Mg^{2+} would preclude the availability of any

nucleotide^{4-} form of the analogues, and thereby account for their inactivity. A corollary to this hypothesis is that CFTR must possess an extremely high affinity for ATP^{4-} because in these and our previously reported experiments (Venglarik et al., 1994), <23 μM ATP^{4-} was present in conditions that maximally stimulated channel activity.

The observation that AMPPNP could not initiate CFTR channel opening parallels the findings of other laboratories (Nagel et al., 1992; Hwang et al., 1994; Gunderson and Kopito, 1994) and indicates that AMPPNP cannot, in any circumstances, interact with a nucleotide binding site on CFTR to initiate channel opening. However, during the course of our studies, Hwang et al. (1994) and Gunderson and Kopito (1994) reported that AMPPNP could lock CFTR in an open state and, as such, does interact with CFTR. The effects of AMPPNP reported by these investigators required that CFTR be in a highly phosphorylated state. This state was defined by a high P_o in the presence of PKA and required the concomitant presence of ATP. Although, Hwang et al. (1994) report that an AMPPNP-sensitive nucleotide binding site can be invoked by highly phosphorylating CFTR with PKA, our results show that the phosphorylation state of CFTR is not the sole determining factor in allowing AMPPNP to alter nucleotide-dependent CFTR gating (see below).

In the present study, the ATP analogue AMPCPP failed to support CFTR channel activity in excised membrane patches ($n = 8$) which is in contrast to an earlier report by Anderson et al. (1991). Other nucleotides reported to support CFTR channel activity (Anderson et al., 1991) were effective in the present system as well e.g., GTP and UTP (Schultz, B. D., unpublished observation). Therefore, the disparity in outcomes for the effect of AMPCPP is not easily explained. Although completed at the same temperatures, differences exist in the conditions employed in these experiments and those of the previous report including the solutions, cell type, expression system, chemical sources, et cetera. Investigations have not yet been undertaken to resolve this discrepancy because of the many possible sources of variation.

Our results are consistent with those described for the permeabilized human sweat duct or T84 tissue systems in that the nonhydrolyzable ATP analogues (AMPPCP, AMPPNP, ATPγS) were unable to activate a previously phosphorylated chloride conductance (Bell and Quinton, 1993; Quinton and Reddy, 1992). Quinton and Reddy (1992) reported that ATP activated an apical membrane chloride conductance in permeabilized human sweat duct. However, the concentrations of ATP required to maintain the chloride conductance (0.5–10 mM) were 10-fold greater than those reported by Anderson et al. (1991), and seventy-fold greater than those we have previously reported (Venglarik et al., 1994) for stimulation of CFTR channel activity in excised membrane patches. AMPCPP and 2-methyl-thio-ATP were capable of increasing sweat duct apical membrane chloride conductance (Quinton and Reddy, 1992). However, the authors did not report the concentration of nucleotide employed or the extent of stimulation to allow for comparison to the present work. AMPPNP and ATPγS increased apical membrane chloride conductance of either sweat ducts or T84 colonic cells, but only in the presence of cAMP and 0.1–1 mM ATP (Bell and Quinton, 1993; Quinton and Reddy, 1992). In these semi-intact systems, ATP could be sequestered at intracellular binding sites or in intracellular

compartments (Dubyakand El-Miatassim, 1993). The addition of the nonhydrolyz-able analogues could then displace or release ATP to increase its effective free concentration and, therefore, account for the nucleotide-dependent change in conductance. Alternatively, ATP analogues may interact with differentially phosphorylated forms of CFTR resulting in the incremental stimulation of channel activity (Gadsby, Hwang, Horie, Nagel, and Nairn, 1993; Hwang, Horie, and Gadsby, 1993). Quinton and co-workers (Bell and Quinton, 1993; Quinton and Reddy, 1992), showed stimulation by nonhydrolyzable analogues only in the continued presence of cAMP which might suggest that a highly phosphorylated, but perhaps less stable form of CFTR is required for effects of ATP analogues to be observed. It is also possible that the ATP analogues may be affecting a regulatory protein present in this semi-intact system or, although highly unlikely, a conductance other than CFTR.

It has previously been shown that changes in the nucleoside portion of the molecule have relatively little effect on the ability to support CFTR channel activity. Purine (guanosine, inosine) as well as pyrimidine (uracil, cytosine, thymidine) substitutions for adenine in ATP result in compounds that support CFTR channel activity (Anderson et al., 1991; Schultz, B. D., unpublished observations). Both ADP and GDP were previously shown to reduce CFTR channel activity, but ADPβS was unable to alter channel activity (Anderson and Welsh, 1992) again showing that an alteration in the phosphate chain precludes nucleotide interaction with CFTR. Thus, CFTR appears to impose few constraints on the nucleoside portion of the interacting ligand. The minimum requirement for a molecule to interact with this stably active form of CFTR appears to be a nucleoside diphosphate containing no ester substitutions because neither AMP nor nonhydrolyzable ATP analogues altered CFTR channel activity.

Effects of AMPPNP, Temperature, and PKA on CFTR Channel Activity

Results we present which show that AMPPNP does not interact with CFTR are distinctly different from those of Hwang et al. (1994) and Gunderson and Kopito (1994) who reported that AMPPNP interacted with CFTR to cause a locked open state. In examining the systems and protocols employed, many explanations for the discrepancies in outcomes were possible. Differences exist in the variant forms of CFTR used, the direction of Cl−flow, the electrochemical potential across the membrane, the ionic composition of the solutions, the presence of heavy metals, temperature, and the presence of PKA to name a few. Since Hwang et al. (1994) reported that a highly phosphorylated form of CFTR was required to see an effect of AMPPNP and since Bijman et al. (1993) reported that reduced temperature altered the ATP dependence of CFTR, we chose to evaluate the effect of these parameters on CFTR channel gating. Results shown in Figs. 9 and 10 and reported in the text demonstrate that neither reduced temperature nor the presence of PKA alone greatly alter the gating characteristics of CFTR. However, when the conditions of both reduced temperature and the presence of PKA were used, CFTR gating changed to a different kinetic state characterized by a very high P_o (~ 0.9). The P_o achieved in our experiments in the presence of PKA at 25°C was equal to or greater than that reported by either Hwang et al. (1994) or Gunderson and Kopito (1994) in the added presence of AMPPNP. Therefore, our observations indicate that the locked open

state does not require the presence of AMPPNP. Instead, PKA appears to induce a kinetic state at 25°C which is not evident at physiologic temperatures. Whether under these conditions a nucleotide binding site is exposed on CFTR or an associated regulatory protein which is physiologically or biochemically relevant and which can interact with nonhydrolyzable nucleotide analogues requires further investigation.
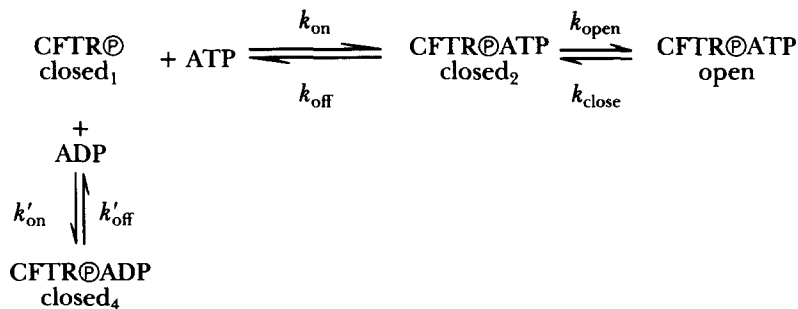
CONCLUSION

In conclusion, the present data show that CFTR channel activity is regulated by the physiologically relevant adenine nucleotides ATP and ADP. The nucleotides appear to have similar affinity for CFTR and thus, the nucleotide binding site of CFTR may act as an energy sensor to reduce chloride transport when cell energy is depleted. The results further show that, at normal human body temperature, ATP analogues with substitutions in the phosphate chain fail to interact with nucleotide binding site of CFTR regardless of the putative phosphorylation state of the protein. Therefore, this binding site must set extreme constraints on only the phosphate moiety of the nucleotide because purine or pyrimidine nucleoside substitutions are permitted. Additionally, we demonstrate that PKA-dependent CFTR regulation is temperature sensitive. Regulatory steps which have been demonstrated at lower temperatures do not perceptibly occur at normal human body temperature.

APPENDIX

Effect of ADP on $2\pi f_c$ and P_o Based on a Simple Competitive Mechanism

To predict and interpret the relationships between ADP concentration ([ADP]), $2\pi f_c$ and P_o it is first necessary to obtain a kinetic model that accounts for CFTR gating. Data presented in the current report are from experiments completed in the absence of PKA indicating that dephosphorylation to the $closed_0$ state of Scheme I or II was not occurring at a perceptible rate during these experiments. Previously we demonstrated that flickery closures of CFTR do not appear to make a significant contribution to P_o (Venglarik et al., 1994), i.e., short-lived closures occur only within an open burst such that the ratio of short-lived closed times to open times is constant (<0.03) and independent of nucleotide concentration (see also Fig. 7). Thus, we can employ a relatively simple four-state mechanism to describe CFTR gating in the presence of ATP and ADP:

$$
\begin{array}{ccc}
\text{CFTR®} & & \xrightarrow{k_{on}} & \text{CFTR®ATP} & \xrightarrow{k_{open}} & \text{CFTR®ATP} \\
\text{closed}_1 & + \text{ATP} & \xleftarrow{k_{off}} & \text{closed}_2 & \xleftarrow{k_{close}} & \text{open}
\end{array}
$$

$$
\begin{array}{c}
+ \\
\text{ADP} \\
k'_{on} \updownarrow k'_{off} \\
\text{CFTR®ADP} \\
\text{closed}_4
\end{array}
$$

SCHEME III

k_{on} and k_{off} are the on and off rate constants for ATP, k'_{on} and k'_{off} are the on and off rate constants for ADP, and k_{open} and k_{close} are the opening and closing rate constants. The other features of this scheme can be identified in the more global descriptions of CFTR gating shown in the Introduction (Scheme I) and Discussion (Scheme II). Using Scheme III it is possible to derive the expected relation between the opening rate (r_+) and [ADP]. Because the closing rate (r_-) is expected to be constant in Scheme III (Venglarik et al., 1994), the equation relating [ADP] to r_+ can then be used to derive the predicted effect of [ADP] on $2\pi f_c$ and P_0 (Colquhoun and Hawkes, 1981, 1983; Venglarik et al., 1994).

Based on rate theory (Colquhoun and Hawkes, 1981; 1983), the opening rate is the product of the opening rate constant (k_{open}) and the probability that a shut CFTR is in the closed$_2$ state [P(C_2| C)]. In the simple competitive Scheme III, $P(C_2|$ C) is given by:

$$P(C_2|C) = \frac{\dfrac{k'_{off}}{k'_{off} + k'_{on} * [ADP]} * k_{on} * [ATP]}{\dfrac{k'_{off}}{k'_{off} + k'_{on} * [ADP]} * k_{on} * [ATP] + k_{off}} \tag{4}$$

Rearranging this expression and substituting dissociation constants (K_D and K_i) for k_{off}/k_{on} and k'_{off}/k'_{on} yields:

$$P(C_2|C) = \frac{\dfrac{[ATP]}{K_D}}{1 + \dfrac{[ATP]}{K_D} + \dfrac{[ADP]}{K_i}}. \tag{5}$$

This equation is similar to the formula used to describe competitive inhibition in Michaelian enzyme kinetics (Segel, 1976).[4] As mentioned previously, the opening rate for CFTR is simply the product of k_{open} and $P(C_2|$ C) (Eq. 4).

The relationship between [ADP] and $2\pi f_c$ is obtained by noting that $2\pi f_c$ is the sum of the opening and closing rates:

$$2\pi f_c = r_+ + r_- \tag{6}$$

Substituting the product of k_{open} and $P(C_2|$ C) (Eq. 5) for r_+ in Eq. 6 yields:

$$2\pi f_c = k_{open} * \frac{\dfrac{[ATP]}{K_D}}{1 + \dfrac{[ATP]}{K_D} + \dfrac{[ADP]}{K_i}} + r_-. \tag{7}$$

[4] The use of this general formula to describe competitive inhibition in enzyme kinetics assumes that the on and off rates are rate limiting for activity. Note that a ligand-activated channel with a $(P_0)_{max} < 1$ clearly violates this assumption and normalizing P_0 by dividing by $(P_0)_{max}$ will not correct this problem (refer to Eq. 10).

Because r_- is expected to be constant in Scheme III, Eq. 7 predicts that $2\pi f_c$ will decrease as a simple inhibition function of [ADP] plus a constant. We found that the plot of $2\pi f_c$ as a function of [ADP] agreed with this prediction but contained a large variance (data not shown, Schultz, Venglarik, Bridges, and Frizzell). However, we were able to reduce this variance by taking the difference between the values of $2\pi f_c$ observed before and after ADP addition ($\Delta 2\pi f_c$) as shown in Fig. 5. This result suggests that there is patch-to-patch variation in the closing rate (open time) but we have not systematically investigated this possibility to identify potential sources of variation. Regardless, the plot of the concentration-response relation of $\Delta 2\pi f_c$ is given by:

$$\Delta 2\pi f_c = (r_+)_{max} * \frac{[ADP]}{K_i * \left(1 + \frac{[ATP]}{K_D}\right) + [ADP]} . \tag{3}$$

Where $(r_+)_{max}$ is the opening rate before ADP addition. Note that $\Delta 2\pi f_c$ is expected to become larger in magnitude as a Michaelis-Menten type saturating function. However, the Michaelis constant $\{ K_i(1 + [ATP]/K_D\}$ in Eq. 3 does not correspond to the ADP dissociation constant (i.e., K_i or k'_{off}/k'_{on}).

The relationship between [ADP] and P_o is obtained based on the observations that k_{open} is rate limiting and that brief shut events do not make a significant contribution to P_o (Venglarik et al., 1994). This simplifies the four-state model to a single set of closed-open transitions. Thus:

$$P_o = \frac{r_+}{r_+ + r_-} \tag{8}$$

Substituting the product of k_{open} and $P(C_2| C)$ (Eq. 5) for r_+ and rearranging the resulting expression yields:

$$P_o = \frac{\dfrac{k_{open}}{k_{open} + r_-} * \dfrac{[ATP]}{K_D}}{\dfrac{[ATP]}{K_D} + \dfrac{r_-}{k_{open} + r_-} * \left(1 + \dfrac{[ADP]}{K_i}\right)} . \tag{9}$$

Because $(P_o)_{max} = k_{open}/(k_{open} + r_-)$ and $(1 - (P_o)_{max}) = r_-/(k_{open} + r_-)$:

$$P_o = (P_o)_{max} * \frac{\dfrac{[ATP]}{K_D * (1 - (P_o)_{max})}}{1 + \dfrac{[ATP]}{K_D * (1 - (P_o)_{max})} + \dfrac{[ADP]}{K_i}} . \tag{10}$$

This equation is also similar in form to the equation used in enzyme kinetics (Segel, 1976). However, the term representing the Michaelian constant for ATP $\{ K_s = K_D *$ $[1 - (P_o)_{max}]\}$ in Eq. 10 accounts for the notion that k_{open} is rate limiting in normal CFTR Cl^- channel gating (Briggs and Haldane, 1921; Venglarik et al., 1994). Substituting the Michaelian constant (K_s) for $K_D * [1 - (P_o)_{max}]$ and adding a term

$[(P_o)_{min}]$ that enabled us to test the hypothesis that all of the P_0 is ADP sensitive yields Eq. 2:

$$P_0 = (P_o)_{max} * \left(\frac{\dfrac{[ATP]}{K_s}}{1 + \dfrac{[ATP]}{K_s} + \dfrac{[ADP]}{K_i}} \right) + (P_o)_{min}.$$

(2)

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