ADP inhibits function of the ABC transporter cystic fibrosis transmembrane conductance regulator via its adenylate kinase activity

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ADP interacts with the nucleotide-binding domains (NBDs) of the cystic fibrosis transmembrane conductance regulator (CFTR) to inhibit its Cl^- channel activity. Because CFTR NBD2 has reversible adenylate kinase activity (ATP + AMP ⇆ ADP + ADP) that gates the channel, we asked whether ADP might inhibit current through this enzymatic activity. In adenylate kinases, binding of the two ADP molecules is cooperative. Consistent with this hypothesis, CFTR current inhibition showed positive cooperativity for ADP. We also found that ADP inhibition of current was attenuated when we prevented adenylate kinase activity with P^1,P^5-di(adenosine-5′) pentaphosphate. Additional studies suggested that adenylate kinase-dependent inhibition involved phosphotransfer between two nucleotide diphosphates. These data indicate that the adenylate kinase reaction at NBD2 contributed to the inhibitory effect of ADP. Finding that ADP inhibits function via an adenylate kinase activity also helps explain the earlier observation that mutations that disrupt adenylate kinase activity also disrupt ADP inhibition. Thus, the results reveal a previously unrecognized mechanism by which ADP inhibits an ABC transporter.

ATPase | chloride channel | nucleotide-binding domain | phosphotransfer

The cystic fibrosis transmembrane conductance regulator (CFTR) anion channel belongs to the ABC transporter family, and it shares the defining features of two membrane-spanning domains and two nucleotide-binding domains (NBDs) (NBD1 and NBD2) (1, 2). In addition, CFTR has a unique regulatory (R) domain. Activity of the CFTR Cl^- channel is controlled by phosphorylation of the R domain and by ATP-binding and enzymatic activity by the NBDs (3–5). Although normal gating requires that ATP interact with both NBDs, they control activity by different mechanisms. At NBD1, ATP binding influences channel opening, but this domain shows little enzymatic activity (6–10). In contrast, at NBD2, both ATP binding and enzymatic activity are key for the normal gating cycle.

Like other ABC transporters, CFTR can function as an ATPase (ATP → ADP + P_i) (11). When ATP alone is present, ATP hydrolysis contributes to channel gating. We recently showed that CFTR also has adenylate kinase activity (ATP + AMP ⇆ ADP + ADP) (12). Moreover, at physiologic ATP and AMP concentrations, this enzymatic activity appears to account for most of the gating. Both the ATPase and adenylate kinase activities share a common ATP-binding site in NBD2, and the presence of AMP determines whether the enzymatic activity is ATPase or adenylate kinase (12).

ADP is well known to inhibit CFTR current (12–17). For example, adding ADP to an equimolar concentration of ATP reduces current by ≈70%. Yet, how ADP inhibits remains uncertain. Some data are consistent with the hypothesis that ADP inhibits by competing with ATP for binding (6, 18, 19). However, knowing that CFTR is an adenylate kinase raised the question of whether this enzymatic activity might be involved in ADP-dependent inhibition. In adenylate kinases, ADP forms ATP and AMP via a readily reversible phosphotransfer reaction (20–22). Although in CFTR there has been no evidence that ADP inhibits via adenylate kinase activity, some data are consistent with this possibility. For example, ADP inhibited current by altering the same gating step as ATP, AMP, and P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A), nucleotides that affect adenylate kinase activity (12, 14, 15, 23). We also found that adding ADP in the presence of ATP generated a pattern of gating consistent with adenylate kinase activity (12). In addition, both adenylate kinase activity and ADP inhibition localize to NBD2 and not NBD1 (12, 13, 24). Finally, there is a correlation between the effect of specific mutations on adenylate kinase-dependent gating and on ADP-dependent inhibition of gating. For example, structural studies predict that the K1250A and D1370N mutations alter the ATP-binding sites, and these mutations disrupted both ATPase activity and adenylate kinase activities, as well as ADP-dependent inhibition. In contrast, the N1303K mutation is predicted to lie outside the ATP-binding site. Consistent with this hypothesis, it had minor effects on ATPase activity and ATPase-dependent gating, yet it disrupted both adenylate kinase activity and adenylate kinase- and ADP-dependent gating (12, 24).

Based on these considerations, we hypothesized that ADP may inhibit CFTR Cl^- current through its endogenous adenylate kinase activity.

Experimental Procedures

Cells and Expression Systems for CFTR. CFTR was transiently expressed in HeLa cells by using a hybrid vaccinia virus system as described (25).

Electrophysiology. We studied CFTR Cl^- channels by using excised inside-out membrane patches. The pipette (extracellular) solution contained: 140 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 5 $CaCl_2$, 100 mM L-aspartic acid, and 10 mM tricine, pH 7.3, with HCl. The bath (intracellular) solution contained 140 mM *N*-methyl-D-glucamine, 3 mM $MgCl_2$, 1 mM Cs EGTA, and 10 mM tricine (pH 7.3), with HCl. After patch excision, channels were activated with catalytic subunit of cAMP-dependent protein kinase (PKA, 80 units/ml) (Promega) and ATP. The cytosolic surface of the patches was continuously perfused with a multichannel rapid change perfusion system. PKA was present in all cytosolic solutions that contained ATP. Holding voltage was −40 mV. Experiments were performed at room temperature (23–26°C).

An Axopatch 200A amplifier (Axon Instruments, Union City, CA) was used for voltage clamping and current recording and the

Abbreviations: NBD, nucleotide-binding domain; CFTR, cystic fibrosis transmembrane conductance regulator; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; PKA, cAMP-dependent protein kinase.

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PCLAMP software package (Version 8.1, Axon Instruments) for data acquisition and analysis. Recordings were low-pass Bessel-filtered at 10 kHz and stored by using a digital tape recorder. Replayed recordings were low-pass-filtered at 100 Hz by using an eight-pole Bessel filter (model 900, Frequency Devices, Haverhill, MA) and digitized at 250 Hz for macropatch recordings.

Reagents. All nucleotides and nucleotide analogs were from Sigma–Aldrich. ATP was added as the Mg^{2+} salt. GDP-NH_2 was an ammonium salt. ADP, GDP, and Ap_5A were used as sodium salts.

Results

ADP Inhibition of CFTR Currents Shows Positive Cooperativity for ADP. When ADP molecules bind adenylate kinases, the enzyme catalyzes transfer of the β-phosphate of ADP bound at the AMP site to the ADP sitting at the ATP site. Binding of the two ADP molecules is cooperative, and the relationship between substrate concentration and product formation is characterized by positive substrate cooperativity (26). This cooperativity was also found in an isolated CFTR NBD2 polypeptide (27).

We reasoned that if intrinsic adenylate kinase activity contributed to ADP inhibition of Cl^- current, then the relationship between the ADP concentration and current inhibition would exhibit positive cooperativity. Consistent with previous reports (13, 15, 17), we found that at a constant ATP concentration, ADP inhibited current in a dose-dependent manner (Fig. 1 *A* and *B*). An Eadie–Hofstee plot of the data revealed a curve convex to the right, a pattern diagnostic of positive cooperativity for ADP (Fig. 1*C*). The Hill coefficient was 1.30 ± 0.04. These results indicate that at least two molecules of ADP interact with CFTR to inhibit current. Thus, they were consistent with the hypothesis that inhibition involves adenylate kinase activity. Alternatively, it was possible that the two molecules of ADP bind cooperatively to the two NBDs. Therefore, to further test whether adenylate kinase activity was involved, we did additional studies.

Inhibition of CFTR Adenylate Kinase Activity Reduces ADP Inhibition. Ap_5A is an adenylate kinase inhibitor (28, 29). In CFTR, Ap_5A-mediated inhibition of gating occurs at NBD2 (12), and in a recombinant NBD2, Ap_5A inhibits both ATPase and adenylate kinase activity (30). We reasoned that if ADP inhibited current solely by competing with ATP, then ADP and Ap_5A would have additive inhibitory effects. On the other hand, if ADP inhibition involved adenylate kinase activity, we predicted that Ap_5A could reduce or eliminate the effect of ADP. Fig. 2 *A* and *B* shows that 15 μM ADP inhibited 35 ± 3% of the current generated by 75 μM ATP; we used 15 μM ADP because it falls on the steep part of the inhibition dose–response curve when channels are exposed to 75 μM ATP. However, with 1 mM Ap_5A, which inhibits ≈50% of the current (12), 15 μM ADP failed to cause additional inhibition. This result is consistent with an ADP action mediated through adenylate kinase activity.

To further test this hypothesis, we asked whether preventing adenylate kinase activity could actually increase current that had been inhibited by ADP. For these studies, we used a low ATP concentration so we could obtain near maximal current inhibition (93 ± 3%) with a moderate amount of ADP (0.1 mM) (Fig. 2 *C* and *D*). We then added a saturating Ap_5A concentration (1 mM) (12) to block adenylate kinase activity. Maximal Ap_5A concentrations inhibit ≈50% of the current (ref. 12 and Fig. 2 *C* and *D*). Under these conditions, adding Ap_5A increased current. Thus, current reduction in the presence of both ADP and Ap_5A was less than with ADP alone. These findings indicate that CFTR adenylate kinase activity contributed to ADP-dependent inhibition.

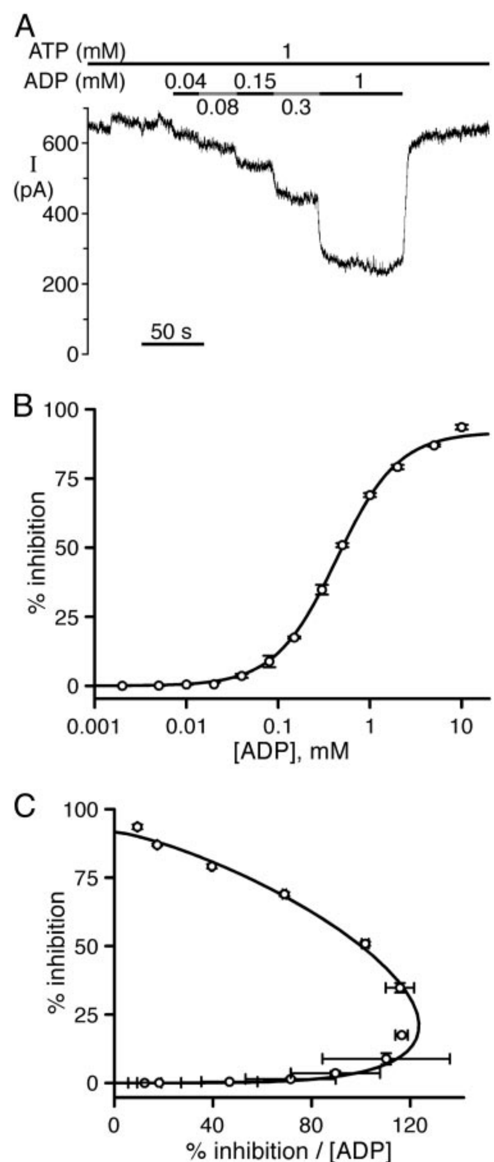

Fig. 1. Inhibition of CFTR Cl^- current by ADP. (*A*) Representative time course of Cl^- current (I) from an excised inside-out membrane patch containing multiple CFTR channels. ATP and ADP were present during times and at concentrations indicated by bars. PKA (80 units/ml) was present throughout. After removing ATP, current returned to a baseline of <2 pA. (*B*) Effect of ADP concentration on CFTR Cl^- current inhibition. Data are from 36 membrane patches. ADP was added to the cytosolic surface in the presence of 1 mM ATP and 80 units/ml PKA. Data are percentage inhibition compared with average of current immediately before and after ADP addition; $n = 4–18$ for each ADP concentration. Line is fit to the Hill equation by using K_m of 0.43 ± 0.01 mM, maximum inhibition of 91.74 ± 0.90%, and Hill coefficient of 1.30 ± 0.04. (*C*) Eadie–Hofstee plot of data from *B*. Pattern with curve convex to the right indicates positive cooperativity.

Induction of Phosphotransfer Enhances Current Inhibition by Nucleotide Diphosphates. Adenylate kinases are usually very specific for an adenine base in their nucleotide monophosphate-binding site, whereas the nucleotide triphosphate-binding site can accept other bases (for review, see ref. 31). For example, GTP can donate a phosphate, but GMP cannot substitute for AMP as a phosphate acceptor. In CFTR, GDP inhibited current, but it failed to produce a gating pattern consistent with adenylate kinase activity, because, although it interacted with the ATP site, it did not bind the AMP site (12). Moreover, the inhibitory

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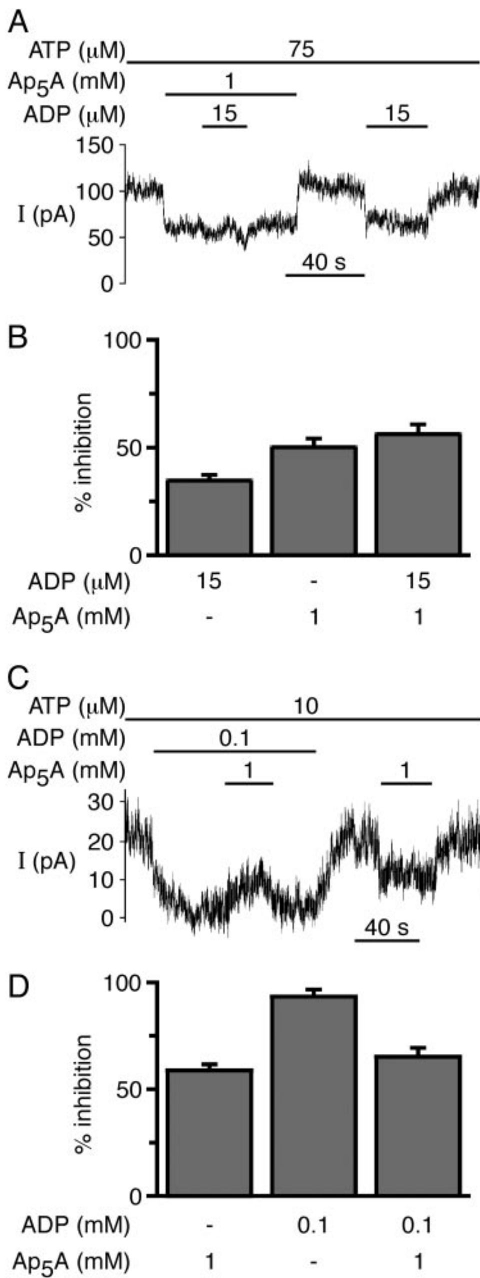

Fig. 2. Inhibition of CFTR Cl^- current by Ap_5A and ADP. (*A*) Representative time course of Cl^- current from an excised inside-out patch containing multiple CFTR channels. ATP, ADP, and Ap_5A were present during times and at concentrations indicated by bars. PKA (80 units/ml) was present throughout. Baseline current after removal of ATP was <1 pA. (*B*) Average data from experiments performed as in *A* with 75 μM ATP. Data are from four membrane patches; $n = 5$ for each condition. (*C*) Representative time course of Cl^- current during exposure to ADP and Ap_5A. Baseline current after removal of ATP was <3 pA. (*D*) Average data in the presence of 10 μM ATP. Data are from four membrane patches; $n = 11$ for each condition.

potency of GDP was lower than that of ADP (12, 13). Therefore, we hypothesized that adding a low concentration of ADP to a high concentration of GDP would enhance current inhibition when ADP bound to an unoccupied AMP site, thereby inducing GDP:ADP phosphotransfer (GDP + ADP → GTP + AMP).

To test this hypothesis, we added concentrations of ADP (1 mM), GDP (2 mM), and GDP-NH_2 (6 mM), all of which inhibited current (generated by 1 mM ATP) to approximately

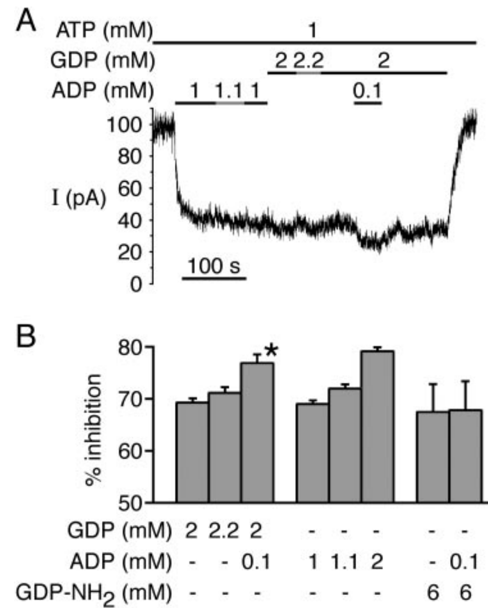

Fig. 3. Induction of phosphotransfer increases GDP inhibition of CFTR Cl^- currents. (*A*) Representative time course of Cl^- current. Baseline current after removal of ATP was <1 pA. (*B*) Average data from 18 membrane patches are shown; $n = 6$–23 for each condition. Asterisk indicates $P < 0.001$ by one-way ANOVA.

the same extent (≈70%) (Fig. 3). We chose nucleotide concentrations that inhibited ≈70% of current, because at that point on the dose-response curve, a small (10%) increase in nucleotide concentration would have a minimal effect unless a mechanism in addition to competition were involved. We found that adding 0.1 mM ADP to 2 mM GDP enhanced current inhibition significantly more than raising the ADP concentration from 1 to 1.1 mM or the GDP concentration from 2 to 2.2 mM. These data indicate that the sites for ADP and GDP in CFTR are not identical. They are also consistent with ADP binding to the ATP and AMP sites but GDP interacting only with the ATP site. Finally, they suggest that the enhanced inhibition generated by adding a small concentration of ADP to GDP resulted from induction of ADP:GDP phosphotransfer.

To further test this hypothesis, we replaced GDP with GDP-NH_2 (guanylyl 5′-phosphoramidate). GDP-NH_2 is a GDP analogue that does not allow phosphotransfer with ADP. Compared with GDP, the relationship between GDP-NH_2 concentration and current inhibition is shifted to the right (data not shown); a potential explanation is that the smaller charge of the GDP-NH_2 molecule may reduce binding affinity. We found that 0.1 mM ADP failed to increase GDP-NH_2 inhibition (Fig. 3*B*). This result indicates that the enhancement of GDP inhibition by ADP involves phosphotransfer, i.e., adenylate kinase activity.

Discussion

ADP reduces CFTR current (12–17). What mechanisms are involved? Earlier work from our laboratory and others suggested that ADP competes with ATP to inhibit activity. For example, ADP reduced [α-^{32}P]8-N_3-ATP photolabeling of CFTR, which is consistent with competition (6, 18, 19). In addition, the base of the nucleoside diphosphate influenced current inhibition in the order ADP > GDP ≈ IDP > UDP > CDP, an order of bases that paralleled that for nucleoside triphosphate stimulation of current (13, 32). This similarity suggested competitive inhibition, although those studies had the limitation that they were performed at only single nucleotide concentrations and did not measure nucleotide binding. Earlier studies showed that ATP

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binding to both NBDs is required for normal gating (3–5, 10, 16), [α-^{32}P]8-N_3-ATP photolabeled both NBDs, and ADP reduced [α-^{32}P]8-N_3-ATP photolabeling of both NBD1 and NBD2 (6, 19). However, it has been difficult to explain the observation that only mutations in NBD2, and not equivalent mutations in NBD1, impaired the ADP inhibition of current (13, 24). This finding suggested a prominent role for NBD2, but it also led us to ask whether processes in addition to competition might be involved. Moreover, because ADP causes a high degree of current inhibition, a model of inhibition based solely on competition rests on the assumption that the affinity of ADP for binding CFTR is apparently higher than that of ATP (33).

Our present data suggest an additional mechanism of inhibition; the reverse adenylate kinase reaction (ADP + ADP $\rightarrow$ ATP + AMP) contributes to the inhibitory effect of ADP. Although we do not know the relative contribution of competition versus adenylate kinase activity in ADP-dependent inhibition, our findings may help explain why mutations in NBD2 that disrupt CFTR adenylate kinase activity interfere with ADP inhibition, but NBD1 mutations do not.

This study also has some limitations. First, we did not measure ADP:ADP phosphotransfer biochemically in full-length CFTR. However, we have shown that a recombinant NBD2 polypeptide had adenylate kinase activity and could produce ATP in the presence of ADP (12). Our earlier work also localized the adenylate kinase gating activity to NBD2, the apparent site at which ADP inhibits. Second, we did not investigate how the phosphorylation state of CFTR influences adenylate kinase-dependent gating and ADP-dependent inhibition. Instead, all our studies used highly phosphorylated channels, i.e., in the continuous presence of PKA. However, earlier work demonstrated that the phosphorylation state influences the response to ATP (34, 35). Although previous studies showed that ADP can also inhibit less highly phosphorylated channels, the mechanisms remain to be examined. Third, we have not yet presented a model of how adenylate kinase gates CFTR. However, so far, no generally accepted model describing the coupling of enzymatic activity to transport has been established for ABC transporters (for review, see ref. 36). Although crystal structures have been reported for several ABC transporter NBDs (9, 37, 38) and two ABC transporters (39, 40), the relationship between structure and function is not understood. In the future, these structures will have to be correlated to enzymatic activity and the conformational changes associated with transport. In this regard, studies of the ABC transporter CFTR have the advantage that its function can be quantitatively assayed with the patch–clamp technique.

Might other ABC transporters also use adenylate kinase activity in cases where energy is not required for transport against an electrochemical gradient? For some ABC transporters, ADP has been shown to potently inhibit ATPase activity (41, 42). Interestingly, in the presence of ADP, downhill substrate transport and ATP formation have been demonstrated for the ABC multidrug transporter LmrA of *Lactococcus lactis* (43). Although ATP may have been synthesized from ADP and P_i, that was not shown directly, and those results were compatible with ATP synthesis via adenylate kinase activity. It will be interesting to investigate whether LmrA also has adenylate kinase activity. Such studies may shed light on the still-unsolved problem of how ABC transporters couple enzymatic activity and substrate transport.

Intracellular ADP concentrations are reported to be 10–26% of the ATP concentration (44–47), and cellular ATP concentrations have been measured from 1 to 11.7 mM in several cells and tissues (48, 49). Therefore, the ADP concentration may lie between 0.1 and 3 mM, concentrations that would affect CFTR currents. By inducing the reverse adenylate kinase reaction under conditions of increased energy demands, ADP could reduce Cl^- currents. Thus, CFTR currents could be coupled to the metabolic state of the cell via mechanisms similar to those proposed for inwardly rectifying K^+ channels (50). It is also interesting to speculate that CFTR adenylate kinase activity could alter ATP, ADP, and AMP levels in a restricted local environment. Perhaps this activity could account for some of the reported effects of CFTR on other membrane transport processes (51, 52).

Knowledge that CFTR has endogenous adenylate kinase activity and that this activity contributes to Cl^- current inhibition may also be of value for future structural studies and for developing CFTR agonists and antagonists, e.g., for the treatment of secretory diarrhea and cystic fibrosis.

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