

CFTR Cl⁻ channel and CFTR-associated ATP channel: distinct pores regulated by common gates

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel that is regulated by phosphorylation of the R domain and ATP hydrolysis at two nucleotide-binding domains (NBDs). It is controversial whether CFTR conducts ATP or whether CFTR might be closely associated with a separate ATP conductance. To characterize ATP channels associated with CFTR, we analyzed Cl⁻ and ATP single channel-currents in excised inside-out membrane patches from MDCK epithelial cells transiently expressing CFTR. With 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath, ATP channels were associated with CFTR Cl⁻ channels in two-thirds of patches that included CFTR. CFTR Cl⁻ channels and CFTR-associated ATP channels had slope conductances of 7.4 pS and 5.2 pS, respectively, and had distinct reversal potentials and sensitivities to channel blockers. CFTR-associated ATP channels exhibited slow gating kinetics that depended on the presence of protein kinase A and cytoplasmic ATP, similar to CFTR Cl⁻ channels. Gating kinetics of the ATP channels as well as the CFTR Cl⁻ channels were similarly affected by non-hydrolyzable ATP analogues and mutations in the CFTR R domain and NBDs. Our results indicate that phosphorylation- and nucleotide-hydrolysis-dependent gating of CFTR is directly involved in gating of an associated ATP channel. However, the permeation pathways for Cl⁻ and ATP are distinct and the ATP conduction pathway is not obligatorily associated with the expression of CFTR.
Keywords: ATP channel/ATP hydrolysis/CFTR/phosphorylation

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the traffic ATPase or ATP binding cassette (ABC transporter) superfamily of proteins and is composed of two membrane-associated domains, each with six putative transmembrane segments, two nucleotide-binding domains (NBDs) and a large cytoplasmic R domain (Riordan *et al.*, 1989; Rommens *et al.*, 1989). Cystic fibrosis, a common lethal genetic disease, is caused by mutations in CFTR (Welsh and Smith, 1993). Despite its structural similarity to transporters, CFTR is a Cl⁻ channel (Anderson *et al.*, 1991b; Bear *et al.*, 1992) whose

activity is under complex regulation by phosphorylation and cytoplasmic ATP (Anderson *et al.*, 1991a; Cheng *et al.*, 1991). Phosphorylation of the R domain by cAMP-dependent protein kinase (PKA) activates the Cl⁻ conductance (Cheng *et al.*, 1991), presumably by inducing conformational changes or modulating electrostatic interactions of the R domain with other parts of the molecule (Rich *et al.*, 1993). The ABC transporters couple ATP hydrolysis at NBDs to the transport of a wide variety of molecules (Higgins, 1992; Gottesman and Pastan, 1993). However, CFTR conducts Cl⁻ ions down an electrochemical gradient, a process which has no obvious need for external energy (Anderson *et al.*, 1991; Cheng *et al.*, 1991; Bear *et al.*, 1992; Welsh *et al.*, 1992). Nevertheless, CFTR hydrolyzes ATP (Li *et al.*, 1996b) and once the R domain is phosphorylated, intracellular ATP interacts with the NBDs to regulate channel activity by nucleotide hydrolysis (Baukrowitz *et al.*, 1994; Carson *et al.*, 1995). Non-hydrolyzable ATP analogues fail to open phosphorylated CFTR Cl⁻ channels that can subsequently be opened by ATP (Carson and Welsh, 1993; Hwang *et al.*, 1994), and they cause CFTR channels to lock into conducting states of markedly prolonged duration (Gunderson and Kopito, 1994; Hwang *et al.*, 1994). ATP hydrolysis therefore appears to contribute to both opening and closing of CFTR channels (Gunderson and Kopito, 1994; Hwang *et al.*, 1994). Recent studies of NBD mutants suggest that ATP hydrolysis at the two NBDs has opposing functions, with NBD1 and NBD2 controlling channel opening and closing, respectively (Carson *et al.*, 1995). On the other hand, a model for CFTR gating in which ATP binding and hydrolysis at NBD2 is the engine that drives the opening and closing has also been proposed (Gunderson and Kopito, 1995).

Electrophysiological analyses of CFTR mutants indicate that residues in the sixth and twelfth transmembrane segments (TM6 and TM12) contribute to the Cl⁻ channel pore of CFTR (Tabcharani *et al.*, 1993; McDonough *et al.*, 1994; Cheung and Akabas, 1996). The CFTR Cl⁻ channel is blocked by diphenylamine-2-carboxylic acid (DPC), which permeates the channel from either side of the membrane (McCarty *et al.*, 1993), and by 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) from the cytoplasmic side (Linsdell and Hanrahan, 1996b). Serine at position 341 (S341) and K335 in TM6, and T1134 in TM12 appear to interact with DPC (McDonough *et al.*, 1994), and R347 in TM6 appears to be a binding site for DIDS (Linsdell and Hanrahan, 1996b). The estimated diameter of the CFTR Cl⁻ channel pore is approximately 0.6 nm, as assessed by permeation analyses (Hasegawa *et al.*, 1992; Cheung and Akabas, 1996; Linsdell *et al.*, 1997), and by accessibilities of blockers to the binding sites (McDonough *et al.*, 1994; Linsdell and Hanrahan, 1996).

Recent studies have suggested that CFTR may also

mediate a cell membrane permeability to ATP (Reisin *et al.*, 1994; Schwiebert *et al.*, 1995; Prat *et al.*, 1996; Pasyk and Foskett, 1997). However, these results are currently rather controversial, because ATP permeability specifically associated with CFTR could not be observed in other studies (Grygorczyk *et al.*, 1996; Li *et al.*, 1996b; Reddy *et al.*, 1996). Furthermore, the size of the ATP anion is larger than the estimated size of CFTR pore (the smallest silhouette dimension is 1.05 nm). Because the bases of these discrepant results are unknown, it is unclear whether CFTR itself conducts ATP, if specific ATP permeation pathways are regulated by CFTR or whether technical aspects have contributed to the variable results. It is necessary to resolve these issues, because adenine nucleotide permeability associated with CFTR is of potential importance in regulating receptor-mediated cell signaling (Hwang *et al.*, 1996), other ion channels (Schwiebert *et al.*, 1995) and glycoprotein processing (Pasyk and Foskett, 1997). Dysfunction of this permeability could therefore contribute to pathogenesis in cystic fibrosis.

In the present work, we have examined the relationship between expression of CFTR and ATP channels and characterized ATP channels associated with CFTR, by investigating the effects of pharmacological reagents and mutations in CFTR. Our results indicate that expression of CFTR results in the appearance of a novel ATP channel with distinct pharmacological properties from CFTR, but that opening and closing of both channels are regulated by common gates in CFTR.

Results

Characterization of the CFTR-associated ATP channel

In excised inside-out membrane patches from MDCK cells transiently expressing CFTR with symmetrical Cl⁻-containing solutions in the bath and pipette, Cl⁻ channels were observed with typical CFTR characteristics, including linear current-voltage (*I/V*) relation and 7.2 ± 0.2 pS ($n=4$) single channel conductance (Figure 1A and C). To determine whether CFTR is associated with an ATP conductance, we carried out experiments using excised membrane patches with 100 mM ATP and 140 mM Cl⁻ in the pipette and bath solution, respectively. Cl⁻ currents and ATP currents were detected at negative and positive membrane potentials, respectively. ATP channels were associated with CFTR Cl⁻ channels in two-thirds of patches ($^{75}/_{117}$) that included CFTR (Table I). Typical ATP channel activities associated with CFTR Cl⁻ channels are shown in Figure 2A. The ATP channels had a slope conductance of 5.2 ± 0.4 pS ($n=5$) between +30 mV and +90 mV, and a reversal potential of -15 mV (Figure 2B). The CFTR Cl⁻ channel slope conductance was 7.4 ± 0.1 pS ($n=5$) between -30 mV and -90 mV, which was similar to that observed when ATP channels were absent (7.2 ± 0.3 pS, $n=3$) (Figure 1B and C) or with symmetrical Cl⁻ solutions (Figure 1A and C). The CFTR Cl⁻ channel had a reversal potential of +60 mV, close to the estimated reversal potential for Cl⁻ (+85 mV). The presence of two reversal potentials suggests that the permeation pathway for ATP is distinct from the Cl⁻ channel in CFTR. Furthermore, fitting of the data over

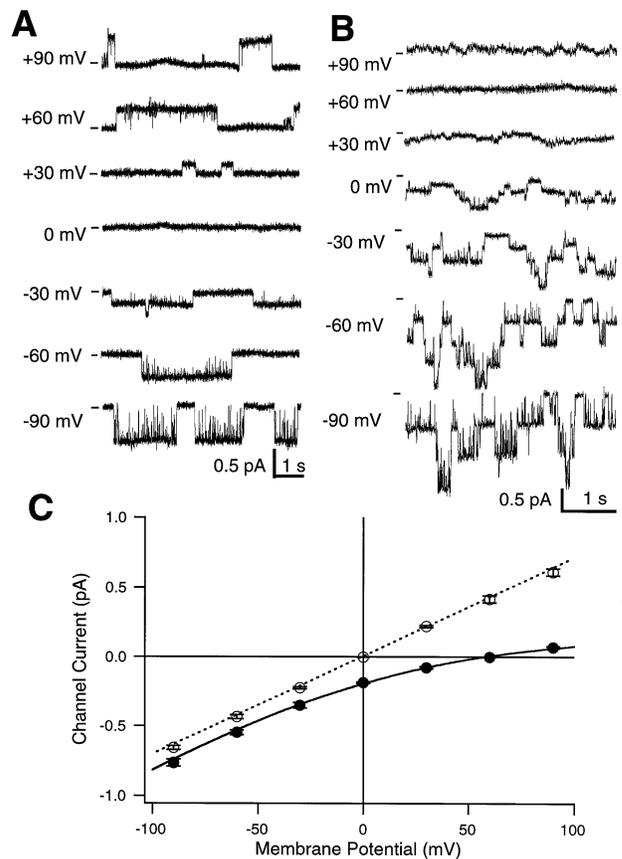


Fig. 1. CFTR Cl⁻ channel activities expressed in MDCK cells. (A) CFTR Cl⁻ channels recorded from an inside-out patch in symmetrical Cl⁻ solutions (140 mM NMDG-Cl) at various membrane potentials. Short lines on the left of current traces indicate the baseline level (channel closed). (B) CFTR Cl⁻ channels, but lack of ATP channels, in an inside-out patch with 100 mM Na₂ATP in the pipette and 140 mM NMDG-Cl in the bath at various membrane potentials. ATP conductances were not associated with CFTR Cl⁻ channels in one third of patches that included CFTR ($^{37}/_{117}$). (C) *I/V* relationship for CFTR Cl⁻ channels with symmetrical Cl⁻ solutions (○, $n=4$ patches) or with 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath (●, $n=3$ patches). The solid line is the best curve fitted to the GHK current equation for Cl⁻ and ATP⁴⁻ with $P_{Cl^-}/P_{ATP^{4-}} = 142.5$ in the asymmetrical condition.

Table I. Frequency of CFTR Cl⁻ channels and ATP channels detected in control and CFTR-transfected MDCK cells

	CFTR-transfected MDCK cell		Control MDCK cell	
	CFTR Cl ⁻ channel (+)	CFTR Cl ⁻ channel (-)	CFTR Cl ⁻ channel (+)	CFTR Cl ⁻ channel (-)
ATP _{CFTR} and ATP _{IND}	7	0	0	0
ATP _{CFTR}	68	0	0	0
ATP _{IND}	5	9	0	5
No ATP channel	37	70	0	19

CFTR Cl⁻ channels and ATP channels were observed in inside-out patches with 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath. The numbers represent frequencies of the presence or absence of CFTR-associated ATP channels (ATP_{CFTR}) and/or CFTR-independent channels (ATP_{IND}) in patches that either included or lacked CFTR Cl⁻ ATP channels. Totals of 196 and 24 patches from CFTR-transfected and control MDCK cells, respectively, were tabulated.

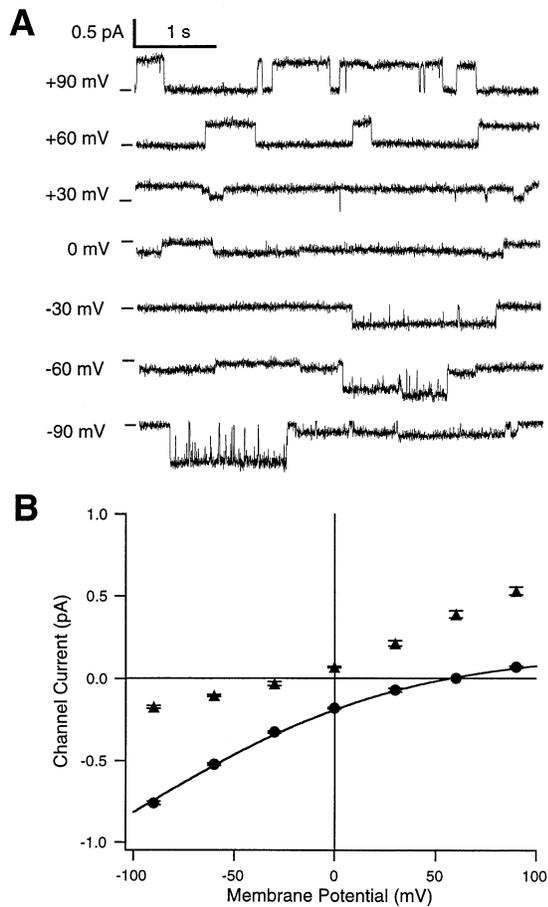


Fig. 2. CFTR Cl⁻ channels and associated ATP channels. **(A)** CFTR Cl⁻ channels (large deflections at -90 mV) and CFTR-associated ATP channels (large deflections at +90 mV and small deflections at -90 mV) in an inside-out patch with 100 mM Na₂ATP in the pipette and 140 mM NMDG-Cl in the bath at various membrane potentials. One hundred nM PKA and 2 mM MgATP were included in the bath solution. Similar ATP channels were associated with CFTR Cl⁻ channels in two thirds of patches that included CFTR (⁷⁵/₁₁₇). Short lines on the left of current traces indicate the baseline level. **(B)** I/V relationship for CFTR Cl⁻ channels (●) and CFTR-associated ATP channels (▲) with 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath (*n*=6 patches). The solid line is the best fit curve for CFTR Cl⁻ channels which is fitted to the GHK current equation for Cl⁻ and ATP⁴⁻ with P_{Cl⁻}/P_{ATP⁴⁻} = 142.5. Fitting of the ATP channel currents yielded P_{Cl⁻}/P_{ATP⁴⁻} = 3, P_{Cl⁻}/P_{ATP³⁻} = 1.6, P_{Cl⁻}/P_{ATP²⁻} = 0.75.

the entire voltage range to one I/V curve with the Goldman-Hodgkin-Katz (GHK) current equation gave poor results. Cl⁻ is excluded as the current carrier at the positive membrane potentials because the reversal potential for Cl⁻ is +85 mV. NMDG is excluded as the current carrier because similar channels were never observed in symmetrical NMDG-Cl solutions. The ATP conductances measured were not due to contaminants within the ATP, since the Na₂ATP employed was 99% pure (Cl⁻ contaminant <0.05%). Therefore, the ~5 pS channel currents were carried by ATP. We also applied rapid voltage steps from -90 mV to +90 mV in order to observe continually the activities of CFTR Cl⁻ channels and CFTR-associated ATP channels. In the example shown in Figure 3, four CFTR Cl⁻ channels and a single ATP channel were observed at membrane potentials of -90 mV and +90 mV, respectively. A similar lack of numerical correspondence between the two channels in this and other experiments

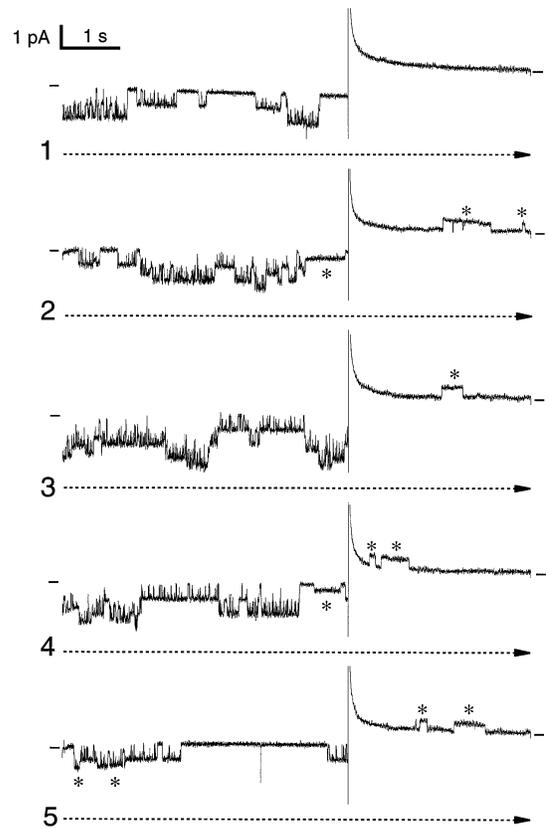


Fig. 3. CFTR Cl⁻ channels and CFTR-associated ATP channels, recorded during rapid voltage steps from -90 mV to +90 mV (at the center line). Five sweeps were continually recorded from a single excised patch. Four CFTR Cl⁻ channels and a single ATP channel were detected at -90 mV and +90 mV, respectively. Short lines beside current traces indicate the baseline level. Asterisks represent open events of the ATP channel.

suggests that the CFTR Cl⁻ channel is not obligatorily linked to the ATP conductance. In some particularly favorable recordings, ATP channels could be observed at -90 mV in current traces containing CFTR Cl⁻ channels (for example, Figure 3). Based on analyses of I/V relations, the larger current deflections corresponded to those expected for the CFTR Cl⁻ channel, whereas the smaller deflections corresponded to those expected for the ATP channel. CFTR Cl⁻ channels had flickery kinetics at negative membrane potentials (Figures 1A, 2A and 3), whereas the ATP channels were not flickery at either positive or negative membrane potentials.

In the other one-third (³⁷/₁₁₇) of patches which contained CFTR, ATP channels were not observed (Table I). In patches that did not contain an ATP channel, Cl⁻ currents were observed at negative membrane potentials, the amplitude of which gradually decreased to zero as the voltage was increased to +60 mV (Figure 1B), close to the estimated reversal potential for Cl⁻ (+85 mV). The relative permeability ratio of the CFTR Cl⁻ channel to Cl⁻ (P_{Cl⁻}) and ATP⁴⁻ (P_{ATP}), calculated by fitting the I/V curve in Figure 1C to the GHK current equation, was P_{Cl⁻}/P_{ATP} ≈ 140, indicating that CFTR is essentially impermeable to ATP. In contrast, fitting of the ATP channel currents yielded P_{Cl⁻}/P_{ATP⁴⁻} = 3, P_{Cl⁻}/P_{ATP³⁻} = 1.6 and P_{Cl⁻}/P_{ATP²⁻} = 0.8 (Figure 2B). Because the predominant ATP

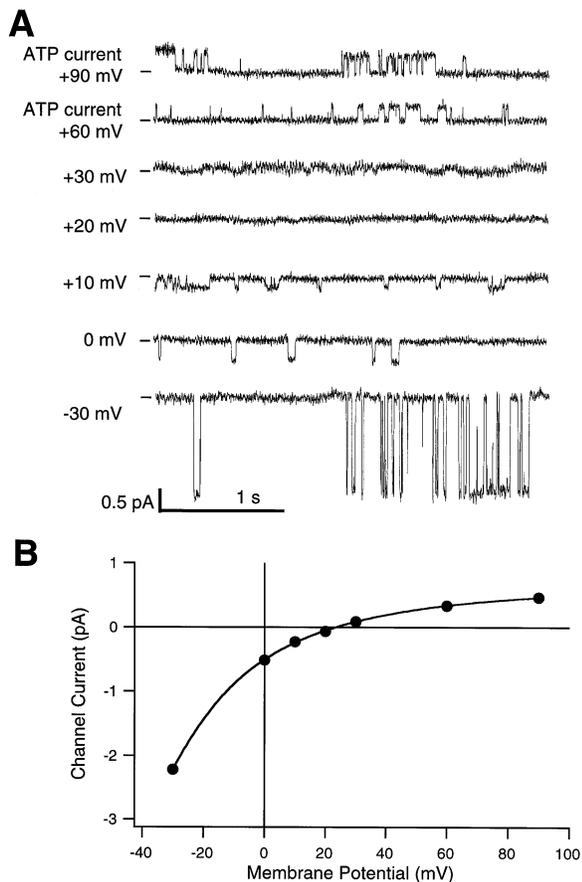


Fig. 4. CFTR-independent ATP conductance in MDCK cells. (A) Endogenous Cl^-/ATP channel activities in an inside-out patch with 100 mM Na_2ATP in the pipette and 140 mM NMDG-Cl in the bath at various membrane potentials. Short lines on the left of current traces indicate the baseline level. (B) I/V relationship for the endogenous Cl^-/ATP channel with 100 mM ATP in the pipette and 140 mM Cl^- in the bath. The slope conductance of the ATP current was 6.3 pS between +30 mV and +90 mV.

ion in our solutions has a valence of ~ -3.5 , the $P_{\text{Cl}^-}/P_{\text{ATP}}$ in our experiments was likely to be ~ 2.5 .

Similar channels were never observed in MDCK cell membrane patches from cells that did not express CFTR. However, in 9 out of 79 patches that did not include CFTR Cl^- channel activities from CFTR-expressing cells, and in 5 out of 24 patches from control cells, different ATP channels (ATP_{ind} channel) were observed (Table I), with a distinct I/V relation and gating kinetics (Figure 4). The slope conductance for ATP was 6.3 pS between +30 mV and +90 mV, but the ATP_{ind} channel, in contrast to the channel associated with CFTR, was substantially more permeable to Cl^- as evidenced by a larger Cl^- conductance at negative membrane potentials, and a reversal potential of $\sim +25$ mV. ATP_{ind} channels were also detected in 12 out of 117 patches that included CFTR Cl^- channels, and in 7 out of 75 patches that included CFTR-associated ATP channels. The frequencies of detecting ATP_{ind} channels in CFTR-transfected cells ($9/79$, $12/117$, $7/75$) are not different compared with that in control cells ($5/24$) (Chi-square test; $p = 0.24, 0.15, 0.13$). Thus, this channel is endogenous to MDCK cells and appears to be unrelated to the expression of CFTR.

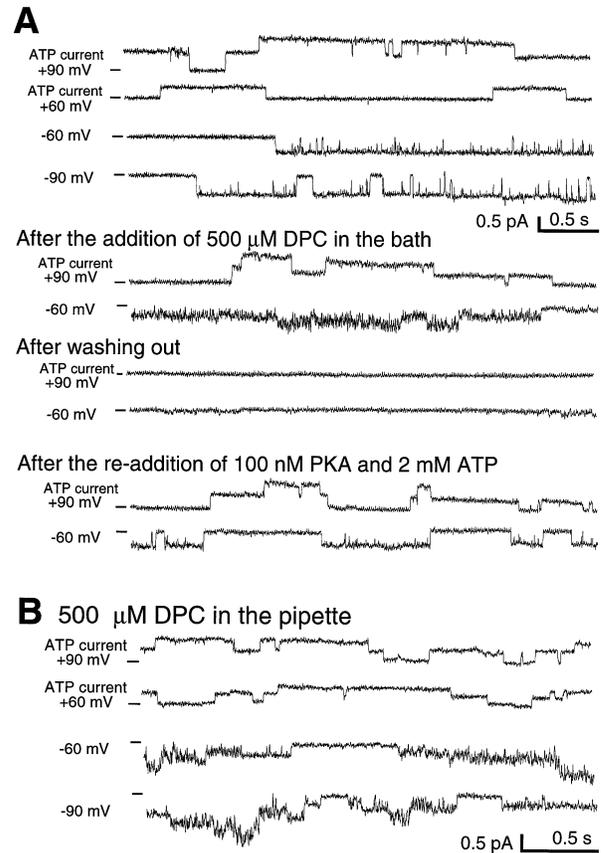


Fig. 5. Effects of DPC on CFTR Cl^- channels and CFTR-associated ATP channels. (A) CFTR Cl^- channels and CFTR-associated ATP channels were recorded in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl^- in the bath at various membrane potentials. 500 μM DPC was added to the bath as indicated (representative of three independently observed channels). Short lines on the left of current traces indicate the baseline level. (B) 500 μM DPC was included in the pipette solution (representative of three independently observed channels).

Relationship between the Cl^- and ATP permeation pathways

To characterize the relationship between CFTR and the CFTR-associated ATP channels, we have compared permeation and gating properties of the two channels in response to pharmacological reagents and mutations in CFTR. To examine the relationship between the two permeation pathways, we compared the effects of Cl^- channel blockers. To analyze the data, the mean open time between blocker-induced rapid closures (mean inter-block open time: t_{io}) of CFTR Cl^- channels and CFTR-associated ATP channels was determined at -90 mV and $+90$ mV, respectively, using a 2 ms delimiter as described in Materials and methods. DPC (500 μM) in either the bath or pipette induced flickery kinetics of the CFTR Cl^- currents ($n=3$; Figure 5A and B), consistent with a rapid channel block as previously observed (McCarty *et al.*, 1993). The addition of DPC to the bath reduced the t_{io} of CFTR Cl^- channels from 207.2 ± 18.1 ms (three patches) to 7.8 ± 0.3 ms (three patches) ($p < 0.01$). In contrast, DPC in the bath or pipette had no effect on the CFTR-associated ATP channels ($n=3$; Figure 5A and B). Glibenclamide (100 μM) also caused a flickery block of CFTR Cl^- channels ($t_{\text{io}} = 15.6 \pm 0.6$ ms, three patches, $p < 0.01$)

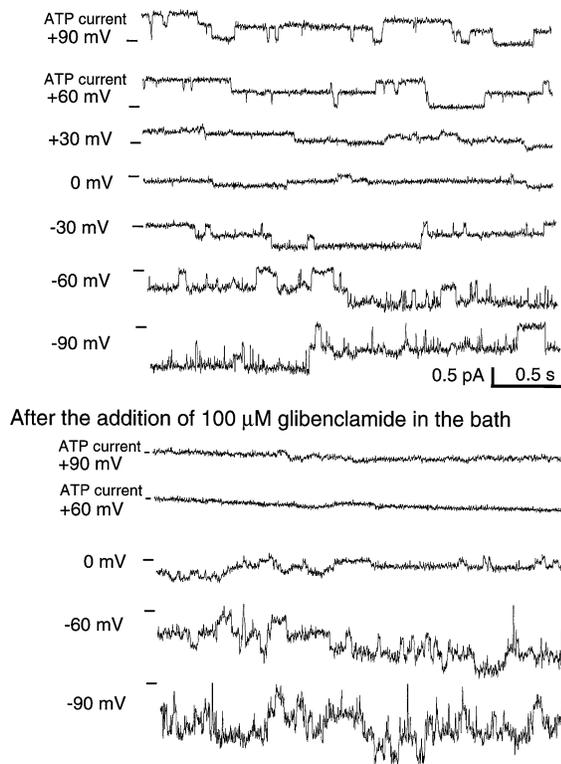


Fig. 6. Effect of glibenclamide on CFTR Cl^- channels and CFTR-associated ATP channels. CFTR Cl^- channels and CFTR-associated ATP channels were recorded in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl^- in the bath at various membrane potentials. 100 μM glibenclamide was added as indicated (representative of three independently observed channels). Short lines on the left of current traces indicate the baseline level.

as previously observed (Schultz *et al.*, 1996; Sheppard and Robinson, 1997), whereas it completely blocked the CFTR-associated ATP channels ($n=3$; Figure 6). Addition of 200 μM DIDS to the bath reduced the open probability (P_o) from 0.41 ± 0.09 to 0.04 ± 0.03 ($n=3$, $p < 0.01$) and induced flickery kinetics in the CFTR Cl^- channels ($t_{io} = 19.5 \pm 1.9$ ms, three patches, $p < 0.01$), in agreement with previous observations (Linsdell and Hanrahan, 1996b). Similar effects were observed for the CFTR-associated ATP channels (control: $P_o = 0.31 \pm 0.06$ and $t_{io} = 623.4 \pm 58.2$ ms; DIDS: $P_o = 0.03 \pm 0.01$ and $t_{io} = 22.4 \pm 1.8$ ms; three patches, $p < 0.01$) (Figure 7A). In contrast, DIDS in the pipette had no effect on CFTR Cl^- channels, whereas it completely blocked the CFTR-associated ATP channels ($n=7$; Figure 7B).

By revealing different inhibitor sensitivities of the two permeation pathways, these results support the biophysical evidence that the CFTR Cl^- channel and CFTR-associated ATP channel pores are distinct. To explore this idea further, we employed site-directed mutagenesis of CFTR. Previous results suggested that residues in the sixth transmembrane segment contribute to the channel pore (Tabcharani *et al.*, 1993; Cheung and Akabas, 1996). Mutations in R347 decrease the single channel conductance of CFTR Cl^- channels and weaken the binding affinity of disulfonic stilbenes for CFTR (Tabcharani *et al.*, 1993; Linsdell and Hanrahan, 1996). We confirmed the reduced single channel conductance of R347E CFTR (1.6 ± 0.1 pS; $n=3$, $p < 0.01$) (Figure 7C). However, the

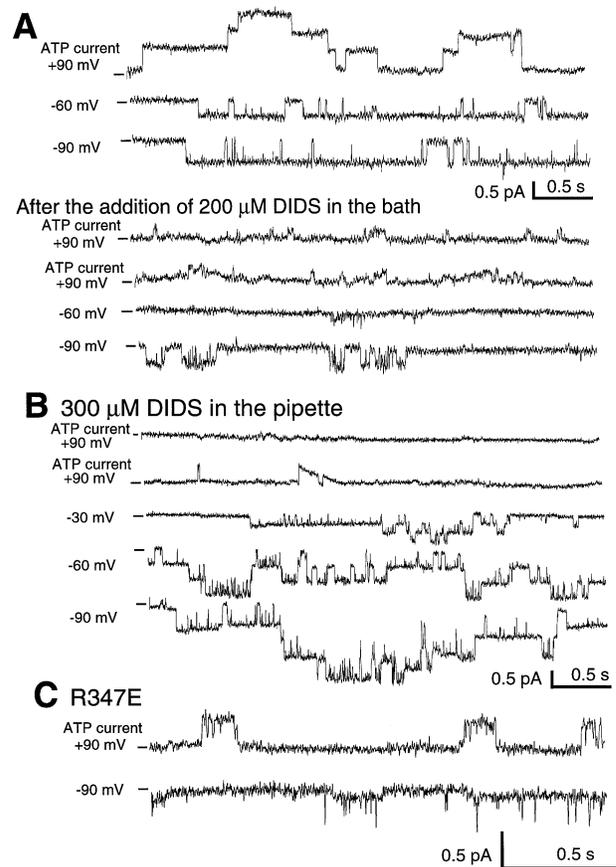


Fig. 7. Effects of DIDS and R347E mutation on CFTR Cl^- channels and CFTR-associated ATP channels. (A) CFTR Cl^- channels and CFTR-associated ATP channels were recorded in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl^- in the bath at various membrane potentials. 200 μM DIDS was added to the bath as indicated (representative of three independently observed channels). Short lines on the left of current traces indicate the baseline level. (B) 300 μM DIDS was included in the pipette solution (representative of seven independently observed channels). (C) Current traces from a MDCK cell expressing R347E in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl^- in the bath.

R347E mutation had little effect on the slope conductance of the CFTR-associated ATP channels (5.08 ± 0.27 pS; $n=3$) (Figure 7C). Thus, the results of pore-directed mutagenesis and pharmacology both suggest that ATP and Cl^- permeate through distinct pathways.

The role of phosphorylation in CFTR-associated ATP channel gating

Although the permeation pathways of CFTR-associated ATP channels and CFTR Cl^- channels are distinct, gating of the two channels appeared qualitatively similar, characterized by slow kinetics. We therefore explored the relationship of the gating behaviors of the two channels in more detail. Both channels required the presence of PKA and cytoplasmic ATP for activity ($n=3$; Figure 5A), suggesting a requirement for phosphorylation. To determine whether the phosphorylation requirement of the ATP channel reflected the phosphorylation requirement of CFTR, or of another protein associated with CFTR, we examined ATP channel activity associated with CFTR mutants that display phosphorylation-independent gating. We first examined the deletion mutant CFTR Δ R-S660A,

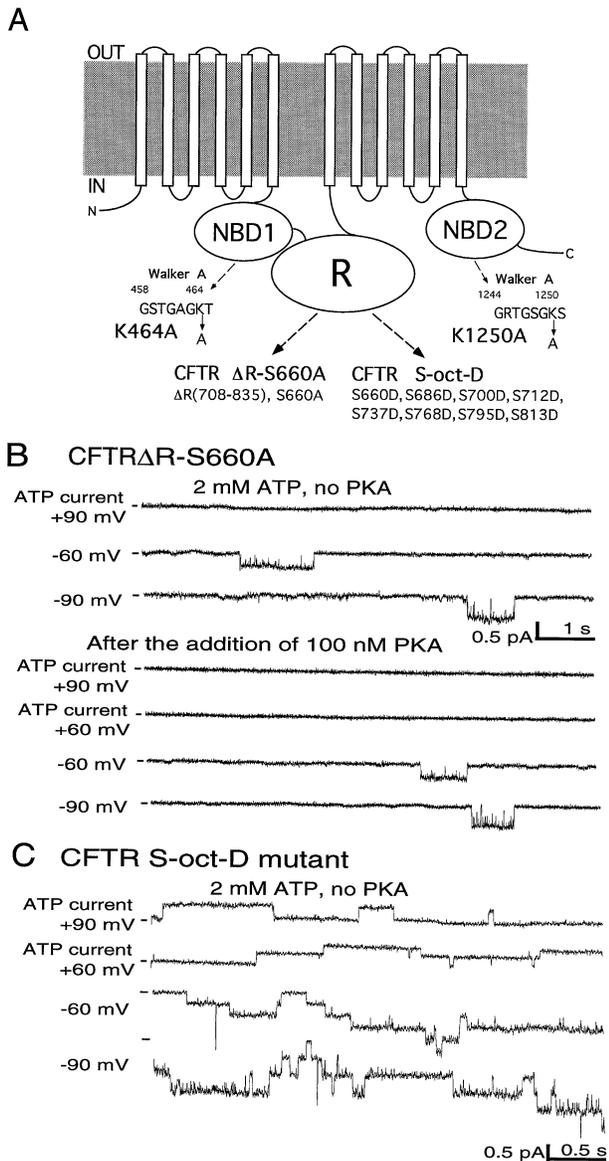


Fig. 8. Effect of the R domain mutations on CFTR Cl⁻ channels and CFTR-associated ATP channels. (A) Diagram of CFTR showing the two membrane-associated domains, two nucleotide-binding domains (NBD1 and NBD2) and the R domain. The residues altered in CFTR Δ R-S660A, CFTR S-oct-D, K464A and K1250A mutants are shown. (B) Current traces from a MDCK cell expressing CFTR Δ R-S660A in the presence or absence of PKA at various membrane potentials (representative of five independently observed channels). Short lines on the left of current traces indicate the baseline level. In eight patches, CFTR Cl⁻ channels were detected without PKA, but ATP channels were not observed. (C) Current traces from a MDCK cell expressing CFTR S-oct-D in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath at various membrane potentials (representative of five independently observed channels). Bath contained 2 mM MgATP but no PKA. ATP channels were associated with CFTR Cl⁻ channels in 5 out of 12 patches that included CFTR.

which lacks much of the R domain and replaces Ser-660 with alanine (Figure 8A). Welsh and co-workers previously showed that the permeation properties of this channel were unaltered, but that its gating was independent of PKA-mediated phosphorylation (Rich *et al.*, 1993). The CFTR Δ R-S660A Cl⁻ channels were active in MDCK cells ($P_o = 0.05 \pm 0.003$, $n=3$) requiring the presence of

cytosolic ATP alone (Figure 8B), and the gating activity did not increase following addition of PKA, in agreement with the previous results. However, the CFTR Δ R-S660A mutant eliminated CFTR-associated ATP channel activities, even in the presence of both PKA and ATP ($n=5$; Figure 8B). This result suggests that the phosphorylation requirement of ATP channel gating was mediated through the CFTR R domain. To examine this further, we expressed CFTR S-oct-D, which contains eight serine-to-aspartate substitutions in the R domain (S660D, S686D, S700D, S712D, S737D, S768D, S795D and S813D) (Figure 8A). Insertion of the negatively charged aspartates in consensus PKA phosphorylation sites in the R domain results in constitutively active CFTR Cl⁻ channels in the absence of PKA (Rich *et al.*, 1993). We found that CFTR S-oct-D generated both CFTR-associated ATP channels and CFTR Cl⁻ channels, which gated without a requirement for PKA-mediated phosphorylation in the presence of cytoplasmic ATP ($n=5$; Figure 8C). The open probabilities of CFTR S-oct-D Cl⁻ channels and associated ATP channels were 0.16 ± 0.03 ($n=4$) and 0.15 ± 0.01 ($n=3$), respectively. Thus, the phosphorylation requirement of ATP channel gating appears to be mediated by the R domain, and the R domain is required for ATP channel activity.

The role of ATP hydrolysis at CFTR NBDs in CFTR-associated ATP channel gating

In addition to phosphorylation of the R domain, gating of CFTR Cl⁻ channels requires nucleotide hydrolysis. To determine whether the ATP channel has a similar requirement, we investigated the effects of hydrolysis-resistant ATP analogues on gating of CFTR Cl⁻ channels and CFTR-associated ATP channels. Adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) alone failed to open either PKA-phosphorylated CFTR Cl⁻ channels, as previously reported, or CFTR-associated ATP channels ($n=4$; Figure 9A). In contrast, ATP γ S or 5'-adenylylimidodiphosphate (AMP-PNP) enabled both CFTR Cl⁻ channels and CFTR-associated ATP channels to remain open, by locking both into prolonged open states ($n=5$; Figure 9B). The mean open times (t_o) of CFTR Cl⁻ channels and CFTR-associated ATP channels, in the presence of 200 μ M ATP and 2 mM AMP-PNP, were 6.5 ± 1.6 s (three patches) and 2.1 ± 0.4 s (three patches), respectively, which were significantly greater ($p < 0.01$) than those of CFTR Cl⁻ channels (1.4 ± 0.3 s, three patches) and CFTR-associated ATP channels (0.7 ± 0.1 s, three patches) with 2 mM ATP. ATP γ S similarly increased t_o of CFTR Cl⁻ channels (6.6 ± 1.3 s, three patches, $p < 0.01$) and CFTR-associated ATP channels (3.6 ± 0.7 s, three patches, $p < 0.01$).

To determine whether this ATP hydrolysis-requirement of ATP channel gating is mediated by the NBDs in CFTR, we investigated the effects of mutations in each NBD. We used mutant CFTRs containing point mutations in the conserved Walker A motifs, which intimately contact the polyphosphoryl moieties of bound nucleoside triphosphates in other ATP- and GTPases. These NBD1 and NBD2 mutants, containing the individual mutations K464A and K1250A, respectively, were expressed in MDCK cells and single-channel currents of CFTR Cl⁻ channels and CFTR-associated ATP channels were analyzed. Compared with the wild-type CFTR Cl⁻ channel,

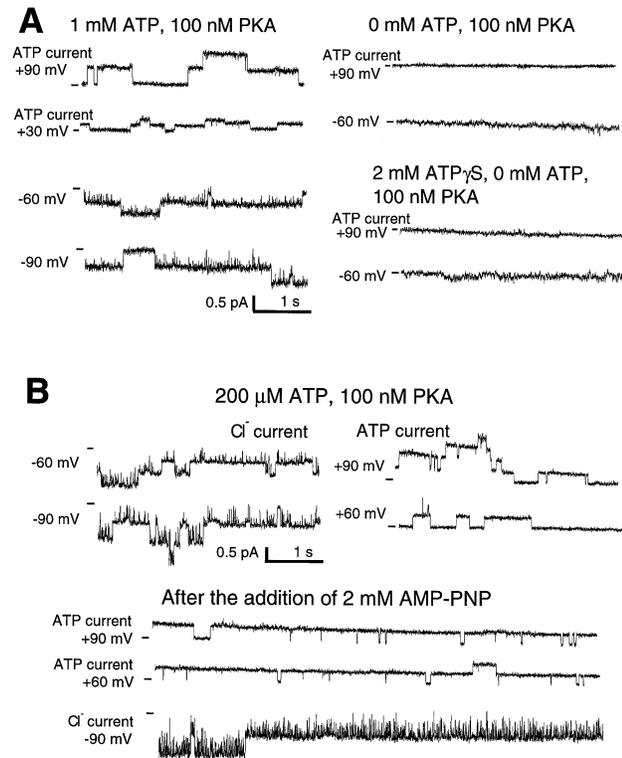


Fig. 9. Effects of poorly-hydrolyzable nucleotide analogues on the kinetics of CFTR Cl⁻ channels and CFTR-associated ATP channels. (A) CFTR Cl⁻ channels and CFTR-associated ATP channels in the presence or absence of ATP followed by addition of 2 mM ATP γ S at various membrane potentials (representative of four independently observed channels). Short lines on the left of current traces indicate the baseline level. (B) CFTR Cl⁻ channels and CFTR-associated ATP channels in the presence of 200 μ M ATP followed by addition of 2 mM AMP-PNP at various membrane potentials (representative of five independently observed channels). ATP γ S had same effects as AMP-PNP ($n=6$).

the K464A mutant exhibited no significant differences in P_o ($p > 0.05$), t_o ($p > 0.05$) and the mean closed time (t_c) ($p > 0.15$), although there was a tendency towards increased t_c and shorter t_o (Figure 10B, C, D and E). Similar, relatively small effects of this mutation have been previously noted (Gunderson and Kopito, 1995). The K464A mutation similarly had no significant effects on the gating of the CFTR-associated ATP channels (Figure 10B, C, D and E). In contrast, mutation of the corresponding lysine in NBD2 (K1250A) resulted in CFTR-associated ATP channels ($p < 0.01$) as well as CFTR Cl⁻ channels ($p < 0.02$) that exhibited significantly prolonged t_o (Figure 10A and D). The K1250A mutation resulted in gating behaviors of wild-type CFTR Cl⁻ channels and CFTR-associated ATP channels which mimicked those induced by non-hydrolyzable nucleotide analogues (Figure 10A). In addition, we noted that the K1250A mutation also decreased t_c of both CFTR Cl⁻ channels ($p < 0.01$) as well as CFTR-associated ATP channels ($p < 0.01$) (Figure 10E). As a result, the K1250A mutation caused a substantial increase in the P_o of both channels ($p < 0.01$) (Figure 10C).

Discussion

Our data indicate that expression of CFTR in MDCK cells induces the appearance of a novel ATP channel. The use

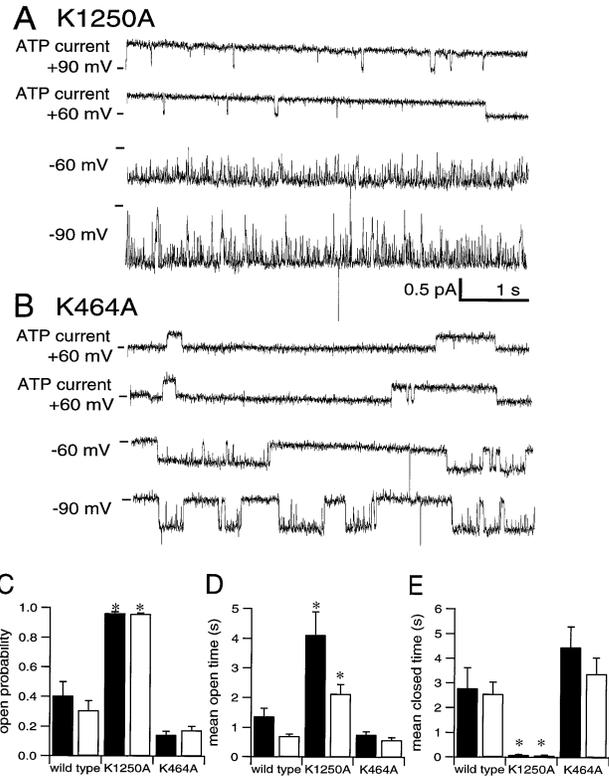


Fig. 10. Effect of mutations of the Walker A motif lysines in NBD1 and NBD2 on the gating of CFTR Cl⁻ channels and CFTR-associated ATP channels. (A) Current traces from a MDCK cell expressing K1250A in an inside-out patch with 100 mM ATP in the pipette and 140 mM Cl⁻ in the bath at various membrane potentials (representative of six independently observed channels). Short lines on the left of current traces indicate the baseline level. ATP channels were associated with CFTR Cl⁻ channels in 6 out of 14 patches that included CFTR. (B) Current traces from a MDCK cell expressing K464A at various membrane potentials (representative of nine independently observed channels). ATP channels were associated with CFTR Cl⁻ channels in 9 out of 17 patches that included CFTR. Comparison of the open probability (C), mean open time (D), and mean closed time (E) of CFTR Cl⁻ channels and CFTR-associated ATP channels in wild-type CFTR and NBD mutants. Closed and open bars represent CFTR Cl⁻ channels and CFTR-associated ATP channels, respectively. The data represent mean \pm SE from three to four patches containing only one active CFTR Cl⁻ channel or CFTR-associated ATP channel. Asterisks indicate statistical significance ($p < 0.05$) relative to wild-type.

of 100 mM ATP to detect ATP currents neither damaged nor permeabilized membrane patches, since stable seals (10–50 G Ω) lasted 15–60 min, the slope conductance of CFTR Cl⁻ channels with ATP in the pipette was similar to that observed in symmetrical Cl⁻ solutions and the activities of CFTR Cl⁻ channels and CFTR-associated ATP channels were regulated normally by the addition of PKA and cytosolic ATP. We noted the occasional presence of ATP currents that appeared to be associated with endogenous Cl⁻ channels in the MDCK cells, but their properties were distinct from those which were associated with CFTR. The frequencies with which these ATP_{ind} channels were detected in CFTR-transfected cells or in patches that included CFTR were not different from the frequency of their detection in control cells, suggesting that they are unrelated to CFTR. ATP_{ind} channels may possibly underlie the CFTR-independent ATP release observed in epithelial cells in response to mechanical

stimuli (Grygorczyk and Hanrahan, 1997) or cell swelling (Wang *et al.*, 1996).

The permeation pathways for Cl⁻ and ATP are distinct

Our results suggest that although CFTR induces the appearance of a different ATP conductance, the novel ATP permeation pathway is not the same pathway that mediates Cl⁻ permeation through CFTR. First, in one-third of patches that included CFTR, CFTR Cl⁻ channels were not associated with detectable ATP conductances. Furthermore, there was no strict correspondence between the numbers of CFTR Cl⁻ channels and ATP channels in the CFTR-containing patches. Second, channel inhibitors that interact with the CFTR Cl⁻ pore had different effects on the Cl⁻ and ATP channels. DPC, which inhibits CFTR Cl⁻ channels from either side of the membrane at hyperpolarizing voltages by causing a rapid flickery block due to binding within the permeation pathway (McCarty *et al.*, 1993; McDonough *et al.*, 1994), was without effect on the ATP channels. Glibenclamide also induces a flickery block of open CFTR Cl⁻ channels (Schultz *et al.*, 1996; Sheppard and Robinson, 1997), but in contrast it completely blocked the ATP channel. Furthermore, DIDS, which cannot permeate through the CFTR pore and causes a voltage-dependent inhibition of CFTR Cl⁻ channels when applied to the cytoplasmic side of the membrane (Linsdell and Hanrahan, 1996b), but is ineffective from the extracellular side, completely blocked the CFTR-associated ATP channel from the extracellular side of the membrane. Third, mutation of a residue in the CFTR Cl⁻ channel pore, R347E, had little effect on the conductance of CFTR-associated ATP channels, in contrast to its effect on the CFTR Cl⁻ conductance. Finally, the two channels exhibited distinct *I/V* relations and reversal potentials. Hysteresis, as sometimes observed with I⁻ present on the extracellular side of the channel (Tabcharani *et al.*, 1997), cannot account for the distinct reversal potentials because the Cl⁻ and ATP channels could be observed simultaneously with conductances consistent with distinct *I/V* relations for the two channels. Taken together, these data suggest that the pore in the ATP channel associated with CFTR is distinct from the pore for Cl⁻ in CFTR.

CFTR gates the CFTR-associated ATP channel

Although our results suggest that separate pores mediate CFTR-associated permeation of Cl⁻ and ATP, gating of the ATP channel is nevertheless closely linked with the gating of CFTR. First, both channels exhibit slow gating kinetics and require cytoplasmic PKA and ATP. Second, the phosphorylation requirement of ATP channel gating is mediated by phosphorylation of CFTR. Thus, CFTR S-oct-D generated CFTR-associated ATP channels as well as CFTR Cl⁻ channels that opened without a requirement for PKA-mediated phosphorylation. Although the mechanisms by which phosphorylation of the R domain influences CFTR Cl⁻ channel gating are still unknown, our results indicate that electrostatic interactions between the R domain and another part of CFTR, or charge-induced conformational changes in the R domain associated with its phosphorylation, enable gating of the CFTR-associated ATP channels as well as CFTR Cl⁻ channels.

The role of ATP hydrolysis in CFTR Cl⁻ channel gating

has been established by analysis of the effects of nucleotide analogues and hydrolysis by-products, and by mutagenesis of the NBDs (Hwang *et al.*, 1994; Carson *et al.*, 1995; Gunderson and Kopito, 1995). These results have been complemented by biochemical evidence of ATP binding (Travis *et al.*, 1993; Logan *et al.*, 1994) and hydrolysis by CFTR (Li *et al.*, 1996b). Mutation analysis suggests that ATP hydrolysis at NBD1 and NBD2 controls both channel opening and closing (Carson *et al.*, 1995; Gunderson and Kopito, 1995). In our experiments, nucleotide hydrolysis appears to be involved in regulating gating of not only the CFTR Cl⁻ channel, but also the CFTR-associated ATP channel. First, ATP γ S alone failed to open either PKA-phosphorylated CFTR Cl⁻ channels or CFTR-associated ATP channels. Second, in the presence of 200 μ M ATP, the poorly-hydrolyzable ATP analogues ATP γ S or AMP-PNP caused both CFTR Cl⁻ channels and CFTR-associated ATP channels to lock into long open states. The similar requirement of ATP hydrolysis for gating of CFTR-associated ATP channels and CFTR Cl⁻ channels suggests that distinct pores of the two channels are both regulated by ATP hydrolysis at CFTR's NBDs. To address this issue, we examined the effects of mutations at NBD1 and NBD2. Mutations in the conserved Walker A motif lysines of NBD1 (K464A) and NBD2 (K1250A) are thought to attenuate ATP hydrolysis with minimal effect on ATP binding (Sung *et al.*, 1988; Schneider *et al.*, 1994; Carson *et al.*, 1995). Previous analyses of the K464A mutant indicated that it had a reduced P_o due to an increased closed time, although these effects were not pronounced (Carson *et al.*, 1995; Gunderson and Kopito, 1995). In our experiments, there was a tendency to lower P_o and longer closed time, but the effects were not significant. However, it is of note that similar effects were observed for the ATP channels associated with this mutant. The K1250A mutation had more pronounced effects. The CFTR Cl⁻ channels exhibited significantly prolonged open times and decreased closed times, resulting in a significant increase in P_o. The prolonged channel open time in the K1250A mutant suggests that ATP hydrolysis at NBD2 closes the channel, consistent with previous observations (Carson *et al.*, 1995; Gunderson and Kopito, 1995). The shortened closed time that we observed has not been noted previously, but suggests that ATP hydrolysis at NBD2 may also play a role in channel opening. Most importantly for the present study, remarkably similar effects on the gating kinetics of the CFTR-associated ATP channels were also observed.

CFTR as an ATP channel regulator

Our results therefore indicate that phosphorylation- and nucleotide-hydrolysis-dependent gating of CFTR is directly involved in gating of an associated ATP channel. Nevertheless, our data also suggest that the permeation pathways for Cl⁻ and ATP are distinct. A model in which distinct pores are regulated by a common gate is reminiscent of the gating behavior of the CIC-0 Cl⁻ channel, in which a slow gate contributed by two CIC-0 monomers simultaneously gates two independent CIC-0 pores (Ludewig *et al.*, 1996, 1997; Middleton *et al.*, 1996). However, the CIC-0 model is distinguished from that for CFTR since the CIC-0 channels are always 'double-barreled', whereas the expression of CFTR is not obligatorily associated with expression of the ATP conduction

pathway. Manifestation of the CFTR-associated ATP conductance may require interaction of CFTR with another molecule(s), although neither the nature of this interaction nor the other molecule(s) are known. The apparent tight coupling between the gating kinetics of CFTR and the ATP channel suggests that the structural changes in CFTR which control the opening and closing of CFTR have similar effects on the ATP conduction pathway. However, our data do not indicate whether the hypothetical interacting molecule(s) contributes, solely or in association with CFTR, to the ATP pore. The interacting molecule(s) may include a separate ATP channel protein regulated by domains in CFTR. Precedents for this mechanism exist in the demonstrations that P-glycoprotein, another ABC transporter protein, regulates distinct cell-volume activated Cl^- channels (Hardy *et al.*, 1995); the interaction of the sulfonylurea receptor (SUR), again another ABC transporter protein, with a K^+ channel is required to manifest ATP-sensitive K^+ channel activity (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995); and the association of CFTR with the epithelial Na^+ channel (ENaC) modifies ENaC gating kinetics and response to phosphorylation (Stutts *et al.*, 1997). Our results suggest an important role for the R domain in contributing to manifestation of the ATP channel, since its deletion, while leaving the CFTR Cl^- channel pore intact, eliminates the ATP channel. It is interesting to note that phosphorylation of the linker region in P-glycoprotein, which is located in an analogous position to the R domain of CFTR, is critically involved in P-glycoprotein regulation of cell-volume activated Cl^- channels (Hardy *et al.*, 1995). Alternatively, the molecular interaction of CFTR with the unknown molecule(s) might create a novel permeation pathway. Finally, the interaction could possibly create an ATP channel within CFTR itself.

Further studies will be required to clarify the nature of the ATP permeation pathway. Because our results suggest that CFTR is a regulator of an ATP channel that is distinct from the Cl^- channel in CFTR, they may provide an explanation for the inability in some studies to observe CFTR-associated ATP permeabilities (Grygorczyk *et al.*, 1996; Li *et al.*, 1996a; Reddy *et al.*, 1996). A CFTR-associated ATP permeability has also been observed in only a subset of CFTR Cl^- conductance-expressing *Xenopus* oocytes (J.Engelhardt, personal communication) and we previously noted variability in the expression of the ATP conductance in different CFTR-expressing CHO cell clones (Pasyk and Foskett, 1997). These observations lend support to the hypothesis that specific molecular interactions with CFTR are required to create the ATP conductance.

Because the ATP concentrations employed in this study exceed physiological levels, future investigations of the concentration–conductance relationship of the channel will be required to determine the magnitude of the ATP permeability associated with this pathway under physiological conditions. Since CFTR is expressed on the apical membranes of epithelial cells, ATP released through the adenine nucleotide permeability associated with CFTR may interact with luminal components, for example purinergic receptors on epithelial cells or on immune cells, for example neutrophils and macrophages associated with the epithelial cell apical membrane surface. Extracellular ATP may stimulate other Cl^- channels in the airway and

play a role in the maintenance of normal fluid balance in the lung (Schwiebert *et al.*, 1995; Hwang *et al.* 1996). Furthermore, since CFTR functions in intracellular organelles as well as in the plasma membrane (Pasyk and Foskett, 1995), the adenine nucleotide permeability associated with CFTR may play important roles in membrane trafficking (Bradbury *et al.*, 1994; Biwersi *et al.*, 1996) and glycoprotein processing (Pasyk and Foskett, 1997). Identification of the molecular basis of the CFTR-associated ATP channel is now of critical relevance for understanding the importance of this permeability in normal and patho-physiology.

Materials and methods

Cells and CFTR expression

MDCK cells were used for transfection and expression of wild-type and mutant CFTR proteins. The cells were grown in MEM (Gibco) supplemented with 10% FCS in 5% CO_2 . They were co-transfected with pcDNA eukaryotic expression vectors containing cDNAs for CFTR and a separate expression vector containing the green fluorescent protein (pGREEN LANTERN-1, Gibco) (10:1) using the Lipofectamine reagent (Gibco), according to the manufacturer's instructions. Expressing cells were identified by visualizing the green fluorescent protein. Cells were plated on glass coverslips the day before their use in patch-clamp experiments and were studied 2 or 3 days after transfection.

Patch-clamp electrophysiology

Excised inside-out membrane patches were obtained by standard techniques. Coverslips containing CFTR-transfected MDCK cells were transferred to a chamber on the stage of an inverted microscope. Unless otherwise specified, the pipette solution contained 100 mM Na₂ATP, 5 mM KCl, 1 mM MgSO_4 , 0.1 mM CaCl_2 , 1.1 mM EGTA and 10 mM HEPES pH 7.3, and the bath solution contained 140 mM N-methyl-D-glucamine chloride (NMDG-Cl), 1 mM MgCl_2 , 0.1 mM CaCl_2 , 1.1 mM EGTA and 10 mM HEPES pH 7.4. 100 nM PKA and 2 mM MgATP were added to the bath for excised membrane patches. Ag/AgCl electrodes were used in the pipette and bath without agar bridges, since the compositions of pipette and bath solutions were not changed during the experiments. Junction potentials were corrected in all experiments by an initial offset applied by the amplifier. Stable seals (10–50 G Ω) were obtained using heat-polished electrodes (tip <0.5 μm diameter) with 20–40 M Ω resistance. Excision of membrane patches did not adversely affect the seal. The membrane potential in excised patches was:

$$V_m = V_{\text{bath}} - V_{\text{pipette}} = V_{\text{cytoplasmic}} - V_{\text{extracellular}} \quad (\text{Equation 1})$$

Positive current flowed from bath to pipette, according to the usual convention. All patch-clamp experiments were performed at room temperature. Single-channel currents were amplified with an Axopatch 200B amplifier with anti-aliasing filtering at 300 Hz, transferred to a Power Macintosh 8100/100 via an ITC-16 interface (Instrutech Corp., Great Neck, NY), digitized at 2 kHz and written directly onto hard disk by Pulse + Pulsefit software (HEKA electronic, Lambrecht, Germany). Channel opening and closing events were detected and analyzed using MacTac 2.0 (Skalar Instruments, Seattle, WA). *I/V* curves were generated from the amplitudes determined by computer-assisted manual measurements of detected events. The membrane potential was stepped from 0 to –90 mV or from –90 to +90 mV in 30 mV increments, or rapidly from –90 mV to, for example, +90 mV (Figure 3). The channel open-state probability (P_o) was determined from amplitude histograms. P_o was measured in patches containing one to six channels and in current recordings of at least 2 min duration. The number of channels was determined as the maximum number of channels open simultaneously throughout the duration of the recording (15–60 min). The mean open time (t_o) and the mean closed time (t_c) were derived from patches with only one active channel (for 15–60 min). Gating of wild-type CFTR is characterized by relatively long openings punctuated by brief closings and separated by relatively long closures. The brief closures are probably due to open-channel block by components of the bath (for instance, HEPES) or membrane (Linsdell and Hanrahan, 1996a; Ishihara and Welsh, 1997) and are therefore not intrinsic parts of the CFTR gating mechanisms. A 40 ms delimiter was chosen to separate openings from brief closings on the basis of the closed dwell time distribution of wild-

type CFTR Cl⁻ channels. Thus, closures shorter than 40 ms are included as part of the open state. In contrast, analysis of channel blocker data employed a 2 ms delimiter. Within single openings from portions of recordings in which only a single open level was observed, the mean open time between blocker-induced rapid closures (inter-block open time, t_{io}) was determined by event-duration analysis of records from patches containing one or two channels and lasting 2–10 min. P_o , t_o , t_c and t_{io} of CFTR Cl⁻ channels and CFTR-associated ATP channels were determined at -90 mV and +90 mV, respectively.

Results are presented as means \pm S.E. of n observations. Unless noted otherwise, statistical significance was determined using unpaired Student's t test. p values <0.05 were considered statistically significant.

Materials

The catalytic subunit of PKA was obtained from Promega. Na₂ATP, MgATP, AMP-PNP, ATP γ S, DIDS and glibenclamide were obtained from Sigma. DPC was from RBI.

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