Commentary

CFTR Channel Gating: Incremental Progress in Irreversible Steps

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Two papers, one in this issue (Weinreich et al., 1999) and the other in the April issue of *The Journal* (Zeltwanger et al., 1999), help clarify the gating mechanisms of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, the products of the gene found mutated in cystic fibrosis patients. CFTR is a most unusual ion channel. It is a prominent member of the ABC transporter superfamily and comprises two homologous halves, each with a probably hexa-helical transmembrane domain followed, in the primary sequence, by a cytoplasmic nucleotide-binding domain (NBD); the two halves are linked by an ~20-kD intracellular regulatory (R) domain loaded with sites that can be phosphorylated by PKA and/or PKC (Riordan et al., 1989). Initially dubbed "regulator" because of its transporter family relatives, and more recently fingered as a bona fide modulator, somehow, of ENaC and possibly other channels and transporters, CFTR is, nevertheless, indubitably an ~10 pS Cl^- channel, albeit with byzantine gating habits (for recent reviews, see Sheppard and Welsh, 1999; Gadsby and Nairn, 1999). Unlike other ion channels, CFTR channels won't open until they've been phosphorylated by PKA, presumably within the R domain, and even then they won't open unless they're supplied with ATP or other hydrolyzable nucleoside triphosphates (Anderson et al., 1991), a result leading to the proposal that channel opening might be energized by ATP hydrolysis. Shortly thereafter, in mixtures of hydrolyzable and nonhydrolyzable nucleoside triphosphates, CFTR channels were found to open but, instead of always closing in 1 s or less as normal, they often became locked in the open conformation for minutes (Gunderson and Kopito, 1994; Hwang et al., 1994), implying that both channel opening and closing might involve ATP hydrolysis. In line with that interpretation, ATPase activity at an appropriately stately pace (comparable with the ~1 s^{-1} channel opening and closing rates) was demonstrated for individual NH_2- (NBD1) and COOH-terminal (NBD2) NBD fusion proteins (Ko and Pedersen, 1995; Randak et al., 1997), as well as for full-length CFTR (Li et al., 1996). Rounding out the picture, NBD1 was tentatively assigned the principal role in channel opening, and NBD2 that in closing, on the basis of the marked prolongation of channel openings seen after mutating the conserved Walker A lysine (believed critical for ATP hydrolysis) in NBD2, K1250, but not after the corresponding mutation of K464 in NBD1, which, if anything, seemed to somewhat slow channel opening (Carson et al., 1995; Gunderson and Kopito, 1995).

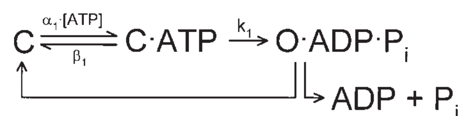
But even this very simple picture overstates the depth of our true understanding of the regulation of CFTR channel gating. The two key questions still await unequivocal answers; i.e., which NBD really subserves which gating function?, and are channel gating and ATP hydrolysis really coupled in the manner just outlined? The difficulties are legion. Among them, gating of CFTR channels seems profoundly influenced by phosphorylation status (more on this later), few point mutants have been examined under more than a single set of conditions, and even the supposedly most promising mutations have defied simple interpretation of their consequences. The CFTR mutants K464A and K1250A, for instance, lie at the heart of challenges to the simple answers to both key questions. Thus, K1250A channels not only close much more slowly than wild-type (WT) channels, they also open more slowly, drawing speculation that ATP binding at NBD2 (rather than hydrolysis at NBD1) might trigger channel opening (Gunderson and Kopito, 1995; compare Sheppard and Welsh, 1999). And recent direct measurements on purified, reconstituted CFTR have revealed virtual abolition of ATPase activity by K1250A, a more than sevenfold reduction of ATP hydrolysis (compared with WT) for K464A, but only an approximately twofold decrement in open probability (P_o) for K1250A channels (because the effect of their markedly slower closing is more than offset by that of their slowed opening) and an even smaller drop in P_o (due to slightly slower opening) for K464A relative to WT (Ramjeesingh et al., 1999), prompting the conclusion that ATP hydrolysis and channel gating are not tightly coupled. As pointed out by Zeltwanger et al. (1999) (compare Gadsby and Nairn, 1999), it is not difficult to explain the K1250A findings, since the very low ATPase activity correlates well with observations of very few openings

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/99/07/49/5 $5.00
Volume 114 July 1999 49–53
http://www.jgp.org

(still conceivably associated with ATP hydrolysis at NBD1) and, after very long open times, an equal number of closings that are presumably associated with dissociation of the ATP, not its hydrolysis, at NBD2. Although it is harder to reconcile the substantially reduced ATPase activity of K464A with its barely altered gating (Ramjeesingh et al., 1999), others have noted that K464A CFTR channels open two- (Gunderson and Kopito, 1995) or fivefold (Carson et al., 1995) more slowly than WT.

Further unraveling of the enigma that is CFTR channel gating is likely to rest on appropriate quantitative or semiquantitative analysis that will require fitting of data to suitable models. Previously published semiquantitative analyses have used linear equilibrium gating schemes not well suited to modeling ATP hydrolysis cycles. On top of this, analysis of gating of CFTR channels is complicated by their burst-like openings, interrupted by brief flickery (intraburst) closures, some nevertheless long enough to be misinterpreted as interburst closures under certain conditions, whose kinetics seem to change irreversibly during the gating cycle (Ishihara and Welsh, 1997; compare Gunderson and Kopito, 1995). Although the brief closures themselves are probably open-channel blocking events unrelated to ATP hydrolysis, because they persist in channels locked open by nonhydrolyzable nucleotides (Gunderson and Kopito, 1994; Ishihara and Welsh, 1997), confusion between longish flickers and true NBD-mediated closings likely accounts for some inconsistencies in reported CFTR gating parameters. A final perfidious difficulty is that analysis of CFTR channel gating is confounded by phosphorylation and dephosphorylation events at multiple sites, with incompletely understood consequences for gating kinetics. Some of the differences in published gating characteristics are almost certainly attributable to variations in channel phosphorylation status.

The work in the two new papers manages to smartly side-step these pitfalls and, together, they resolve some of the outstanding issues regarding function of CFTR's two NBDs. First, semiquantitative analysis in both papers sheds light on the mechanism by which one of the NBDs, presumably NBD1, governs channel opening. Echoing earlier work (Venglarik et al., 1994; Winter et al., 1994), Zeltwanger et al. (1999) show that the opening rate (after prudently ignoring closures $<$80 ms) saturates at high [ATP], indicating that, under those conditions, a step after nucleotide binding, presumably hydrolysis, limits the rate of channel opening. Further analysis by Zeltwanger et al. (1999) revealed that the distribution of the shut lifetimes contains a negative exponential component (i.e., the probability density function, pdf, shows a maximum) due to a deficiency in very brief ($<$200 ms) events. This is inconsistent with a linear equilibrium gating scheme, implying instead a cyclic mechanism (Scheme I) with irreversible steps (Colquhoun and Hawkes, 1995).

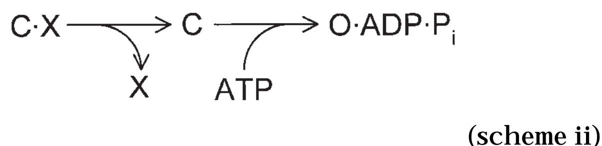


(scheme i)

The paucity of brief closures occurs because the strict forward cycling ensures that during every shut event a channel has to transit through two states (C and C·ATP) before it can reopen. The cyclic gating scheme of Zeltwanger et al. (1999) describes channel opening in terms of three rate constants, α_1, β_1, and k_1, and predicts the simple Michaelis-Menten type dependence of the opening rate on [ATP] seen in the data. A Michaelis-Menten fit yields two parameters, namely the rate of opening at saturating [ATP], given by k_1, and the [ATP] supporting half-maximal opening rate, $K_{0.5} = (\beta_1 + k_1)/\alpha_1$. The latter formula provides a linear constraint between β_1 and α_1 (via $\beta_1 = \alpha_1 \cdot K_{0.5} - k_1$), but doesn't permit determination of the individual α_1 and β_1 rate constants. Additional information on α_1 and β_1 is contained in the shut time pdf, which also depends on all three rates. So, in principle, fitting the pdf to extract β_1 (with k_1 fixed and α_1 given by the above linear constraint) would provide a means for estimating α_1 and β_1, but the slow gating of CFTR makes it hard to collect enough events for a reliable fit, and this difficulty is exacerbated by the need to discard very brief events to avoid inclusion of irrelevant (for this purpose) flickery closures.

Weinreich et al. (1999) nicely complement these steady state single-channel measurements by analyzing macroscopic currents under pre–steady state conditions when, after step changes in applied nucleotide concentrations, CFTR channels relax to a new equilibrium, yielding current changes with (multi-) exponential time courses. The time constants of those exponentials provide valuable information about the channel gating parameters. A finding of Weinreich et al. (1999) with important mechanistic implications is that preincubation of closed channels with ADP or the nonhydrolyzable ATP analogues AMP-PNP or NPE-ATP (caged ATP), none of which support channel opening, nevertheless influences, by delaying and slowing, subsequent activation by ATP following a rapid switch to ATP-only solution. That delay is attributable to the waiting time for dissociation of ADP or the other analogues since only then can ATP bind and open the channel. This implies that all those nucleotides can bind to the closed channels at the very site (presumably NBD1), where ATP would normally bind and be hydrolyzed to allow channel opening, and is consistent with previous steady

state analyses of competitive inhibition by ADP of CFTR channel opening by ATP (Schultz et al., 1995; compare Anderson and Welsh, 1992; Gunderson and Kopito, 1994; Winter and Welsh, 1994). A model in which, for simplicity, the ATP binding and subsequent hydrolysis steps of Scheme I are pooled into a single step (Scheme II) predicts two exponential components for the current relaxation.



(scheme ii)

One of the time constants is the inverse of the rate of dissociation of the competitor X (here ADP, AMP-PNP, or NPE-ATP), analogous to the rate β_1 above (Scheme I). The second time constant is the one normally observed for channel opening by the sudden addition of ATP alone, without prior exposure to a competitor. The relative amplitudes and signs of the two components depend on the initial partitioning of the channels between states C·X and C. If all the channels start from C·X, as seems likely for ADP, the sign of the faster exponential component with the "normal" opening time constant becomes inverted, resulting in an initial delay ($dI/dt = 0$ at $t = 0$), followed by a single rising component governed by the rate of dissociation of the competitor. The data of Weinreich et al. (1999) are in good agreement with the above model, and fitting the currents provided estimates of "β_1" that were found to vary considerably for the different nucleotides, 0.05 s^{-1} for AMP-PNP and 0.4 s^{-1} for ADP, in contrast to the >1 s^{-1} for ATP estimated by Zeltwanger et al. (1999). Taken at face value, this comparison implies that some caution is warranted when extrapolating to ATP the absolute values of various rate constants obtained using other nucleotides, but it by no means diminishes the importance of these analogues, and the above approaches, as valuable tools for extracting essential qualitative information on the complex gating mechanism of CFTR channels. For example, previous lack of a clear demonstration of this competitive effect of AMP-PNP at (presumably) NBD1, although recently suggested by an analysis of rates of AMP-PNP–mediated locking at different [ATP] (Mathews et al., 1998), had been used to argue that CFTR's NBDs do not interact with AMP-PNP (Schultz et al., 1995).

Both new papers also provide novel information about how CFTR channels close. Zeltwanger et al. (1999) examined K1250A CFTR channels and found, at millimolar ATP, the extremely long open times (mean ~3 min) reported by others, but, at 10 μM ATP, only the same brief openings (mean ~250 ms) observed for WT CFTR at low [ATP]. They reasoned that if the long openings at millimolar ATP reflect its long dwell time (i.e., tight binding) at the mutated NBD2, which can't hydrolyze it (Ramjeesingh et al., 1999; compare Dousmanis et al., 1996a), then the lack of influence of the mutation on the brief openings at low [ATP] suggests that those openings involve neither binding nor hydrolysis of ATP at NBD2. In other words, the brief openings of both WT and K1250A CFTR channels are interpreted as simply reflecting ATP binding and hydrolysis, and dissociation of the hydrolysis products, at NBD1. For WT channels, Zeltwanger et al. (1999) found an increased mean open time at high [ATP], consistent with stabilization of the channel open state by ATP binding at NBD2, where its subsequent hydrolysis allows the channel to close. This modulation of open duration by [ATP] is reminiscent of that mediated by incremental phosphorylation by PKA previously described for CFTR channels in cardiac my-

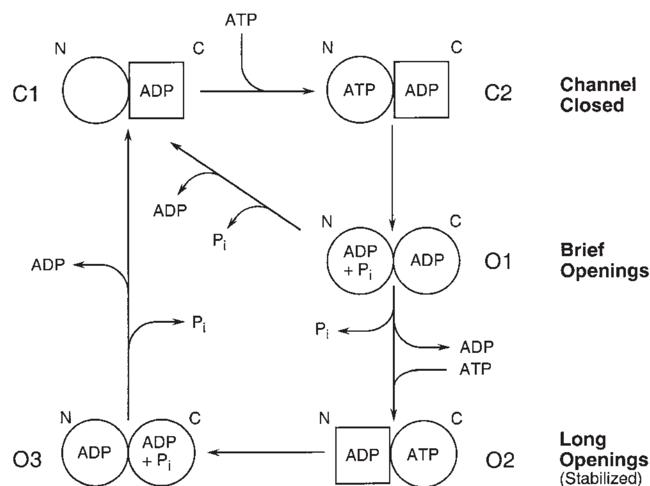


Figure 1. Hypothetical, simplified, cyclical gating scheme for CFTR channels, emphasizing sequential interactions between the NBDs modeled on interactions between G proteins and their cognate dissociation inhibitors and exchange factors. NBD1 and NBD2 are represented as freely accessible (circles) for binding or release of nucleotide, or closed (squares), entrapping the ATP hydrolysis product ADP. In a highly phosphorylated CFTR channel, hydrolysis of ATP at NBD1 (C2 → O1) is proposed to open both the Cl^- ion pore and the NBD2 catalytic site, permitting dissociation of the ADP from NBD2 and binding of ATP. Tight binding of ATP at NBD2 is then proposed to close the NBD1 catalytic site around its hydrolysis product ADP, stabilizing the channel open state (O2). ATP hydrolysis at NBD2 (O2 → O3) opens NBD1, permitting the release of ADP from NBD1 postulated to presage channel closure (O3 → C1), which is proposed to trap the new ADP in NBD2. The diagonal arrow (O1 → C1) results in the abbreviated openings (O1 → C1 faster than O1 → O2 → O3 → C1) seen in poorly phosphorylated channels (or at very low [ATP]). Though drawn as irreversible, for simplicity, nucleotide binding/dissociation reactions are assumed reversible and separate from consequent conformational changes; interactions with nonhydrolyzable nucleoside triphosphates are not included. (Modified from Gadsby and Nairn, 1999.)

ocytes (Hwang et al., 1994; Dousmanis et al., 1996b). The suggestion was made then that the brief openings of partially phosphorylated channels did not involve nucleotide occupancy of NBD2, an interpretation based partly on the failure of such channels to become locked open by AMP-PNP. The simplified model of CFTR channel gating in Fig. 1 shows two gating cycles, a briefer one (C1 → C2 → O1 → C1) involving ATP binding and hydrolysis at NBD1 exclusively, and a longer one (C1 → C2 → O1 → O2 → O3 → C1) that additionally involves ATP binding and hydrolysis at NBD2 (Gadsby and Nairn, 1999). The model can account for all the findings of Zeltwanger et al. (1999) and differs from the scheme proposed by them and by Hwang et al. (1994) only in discounting rebinding of ATP at NBD2 after hydrolysis of the ATP initially bound there. To our knowledge, no presently available data permit a distinction between these two schemes.

It remains unclear whether the scheme in Fig. 1 can account for another telling observation made by Weinreich et al. (1999); namely, the accelerated closing of open CFTR channels upon withdrawal of ATP in the presence of ADP or in exchange for ADP. This finding means that ADP must be able to bind to an already open channel and speed its closure. But does this ADP bind to NBD1 or NBD2? Interruption of ATP hydrolysis cycles by tight binding of inorganic phosphate (P_i) analogues like orthovanadate and beryllium fluoride, that form a stable complex with ADP in the catalytic site, can occur only after the hydrolysis event and dissociation of P_i, but before dissociation of the ADP. These analogues abolish ATPase activity of other ABC transporters like P-glycoprotein (Sankaran et al., 1997), and lock CFTR channels in the open state, even when those channels are poorly phosphorylated and display brief openings and a low P_o (Baukrowitz et al., 1994), interpreted to reflect nucleotide binding and hydrolysis solely at NBD1 (see above). If that interpretation is correct, it suggests that it is the dissociation of ADP, not the earlier release of P_i, that terminates those brief openings and, hence, that the acceleration of closing seen by Weinreich et al. (1999) could not reflect ADP binding at NBD1 on an open channel, but must occur at NBD2. The scheme in Fig. 1 would, indeed, predict a shorter mean open time in the presence of ADP than in its absence due to increased steady state occupancy of the O1 state (and hence a greater fraction of more rapid O1 → C1 closings) at the expense of the O2 state, resulting from competition between ATP and ADP for binding to NBD2. If the binding of ATP to NBD2 were reversible, then sudden addition of ADP upon withdrawal of ATP could also populate the O1 state and so favor fast O1 → C1 closings. In reality, both of these mechanisms might apply, since some brief period of mixing of the two nucleotides must be expected in the solution layer adjacent to the membrane patch. Strictly, such effects ought to be visible as ADP-induced changes in the relative amplitudes of the two exponentially decaying components of macroscopic current during the closing relaxation, or of the two exponential components of the distribution of single-channel open times. Separation of the latter two components was apparently not feasible in the experiments of Zeltwanger et al. (1999), though increases in mean open time were observed as [ATP] was raised, consistent with a greater proportion of longer closings via O2 and O3. The ADP-mediated acceleration of closing (Weinreich et al., 1999) might be explained by the above mechanisms causing a shift from largely slower (O2 → O3 → C1) closings to predominantly faster (O1 → C1) closings on addition of ADP. On the assumptions of instantaneous solution exchange and irreversibility of ATP binding to NBD2, however, Weinreich et al. (1999) propose that NBD2 remains empty in closed CFTR channels as well as in an O1-like state, to which ADP may then bind to yield an O3-like state: acceleration of closing by ADP can then be explained if that O1 → O3 → C1 route is faster than either of the pathways, O1 → C1 or O1 → O2 → O3 → C1, for normal closing in the absence of ADP. Distinguishing among these various alternatives will require accurate separation of the individual exponential components, which is likely to remain difficult until additional control is gained over the phosphorylation and dephosphorylation reactions. Needless to say, all these present models of CFTR channel gating are gross oversimplifications awaiting clarification and amplification constrained by further semiquantitative analysis of kinetic data. And the recent suggestion (Zerhusen et al., 1999) that CFTR normally gates as a dimer, with four NBDs in the functional unit, has now put a newer, bigger cat among the pigeons!

The writing of this commentary was supported by National Institutes of Health grants DK-51767 and HL-49907. László Csanády is a William O'Baker Graduate Fellow at The Rockefeller University.

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