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Biochimica et Biophysica Acta 1461 (1999) 263–274

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Differential function of the two nucleotide binding domains on cystic fibrosis transmembrane conductance regulator

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Received 31 August 1999; accepted 1 September 1999

Abstract

The genetic disease cystic fibrosis is caused by defects in the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). CFTR belongs to the family of ABC transporters. In contrast to most other members of this family which transport substrates actively across a membrane, the main function of CFTR is to regulate passive flux of substrates across the plasma membrane. Chloride channel activity of CFTR is dependent on protein phosphorylation and presence of nucleoside triphosphates. From electrophysiological studies of CFTR detailed models of its regulation by phosphorylation and nucleotide interaction have evolved. These investigations provide ample evidence that ATP hydrolysis is crucial for CFTR gating. It becomes apparent that the two nucleotide binding domains on CFTR not only diverge strongly in sequence, but also in function. Based on previous models and taking into account new data from pre-steady-state experiments, a refined model for the action of nucleotides at two nucleotide binding domains was recently proposed.

Keywords: Chloride channel; Phosphorylation; Pre-steady-state; Gating; Kinetics; Regulation

Contents

Abbreviations: ABC, ATP-binding cassette; BLM, planar black lipid membrane; PKA, cAMP-dependent protein kinase catalytic subunit; Pgp, P-glycoprotein

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00162-5

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) was cloned and sequenced a decade ago in a collaborative effort of three laboratories [1–3]. It was the first gene to be cloned by positional cloning and, although it could not be known at the time from the sequence alone, the second chloride channel cloned (after the glycine receptor [4]). The initial suggestion that CFTR might be a chloride channel [3] was met with great reservation [5] because its primary sequence placed it into a family of active transporters [3], later called traffic ATPases [6], TM6-NBF superfamily [7], or, as best known nowadays, ABC transporters [5].

Overwhelming evidence in the years 1991 and later proved that CFTR itself functions indeed as a phosphorylation- and nucleotide-regulated small-conductance chloride channel. This function is detailed in many exhaustive reviews [8–11], most recently in special issues, of the Journal of Bioenergetics and Biomembranes (e.g. [12]) and of Physiological Reviews (e.g. [13–15]).

That CFTR is also a regulator of epithelial sodium channels was shown first by Stutts et al. [16]. This channel-regulating function of CFTR was later confirmed by others who also found CFTR to regulate many more channels which is again covered in depth in recent reviews [17,18].

Because CFTR is the topic of another article in this special issue [19] the following short review will presuppose knowledge of the structure of CFTR. I will focus on the nucleotide-mediated gating of CFTR chloride channels and its modulation by phosphorylation of CFTR.

2. Phosphorylation activates CFTR chloride channels

The chloride channel activity of CFTR was studied by different electrophysiological techniques, the most important of which are patch clamp studies in the whole cell-, cell-attached patch-, or excised patch-mode and single-channel studies after reconstitution in planar black lipid membranes (BLM). When CFTR was expressed in non-epithelial cells, activation of cAMP-dependent protein kinase (PKA) caused the appearance of a chloride conductance [20,21]. The single-channel conductance of the underlying channels was found to be about 10 pS [20], identical to that of endogenously expressed chloride channels in human epithelial cells [22] expressing CFTR. The final proof that CFTR is a chloride channel was its purification and reconstitution in BLM, resulting again in PKA- and ATP-activated low-conductance chloride channels [23].

The same channels were also found endogenously expressed in rat [24] and human [25] pancreatic duct cells, as well as in heart cells from guinea pig ventricle [26,27] where CFTR expression, albeit weak, was shown by Northern blotting [27]. Fig. 1 exemplifies the activation of cardiac CFTR channels by PKA and ATP. The beginning of the current trace shows that ATP has no effect on non-phosphorylated channels, but addition of PKA catalytic subunit together with ATP leads to a stepwise current increase which persists upon removal of PKA.

Under physiological conditions, the activation by PKA is counteracted by protein phosphatases so that inactivation of PKA is followed by inactivation of CFTR chloride conductance [24,26]. The fact that this is different under the in vitro conditions of an

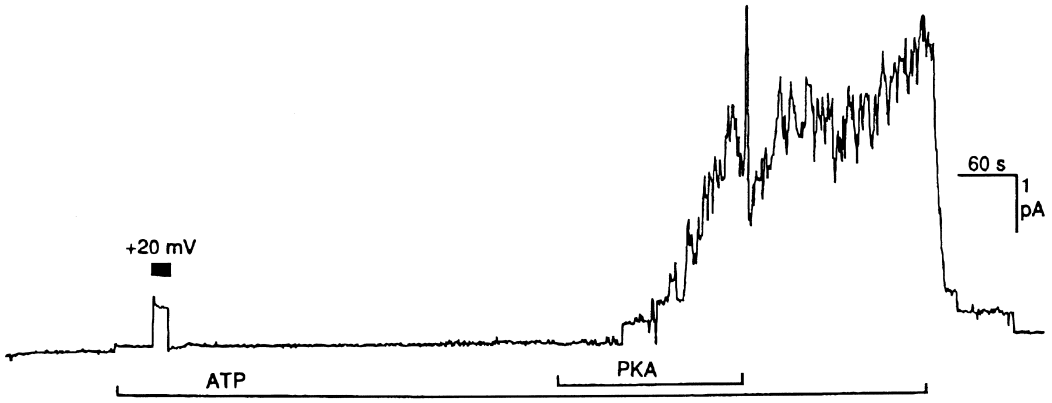

Fig. 1. Activation of CFTR by PKA and ATP. Reprinted with permission from Nagel et al. [27]. Copyright (1992) Macmillan Magazines Limited.

excised inside-out membrane patch indicates that phosphatase activity is relatively low, although it must be stressed that, depending on the expression system and the individual membrane patch, residual protein phosphatase activity is evident (see e.g. 'rundown' in Fig. 2). This protein phosphatase activity must be membrane-bound as it is also seen under conditions of vigorous solution exchange (like in Fig. 2). The participation of protein phosphatases in CFTR channel activity rundown was shown by its reversal with renewed application of PKA (e.g. [28,29]). The fast inactivation upon removal of ATP, seen in Fig. 1, however, is not related to lacking phosphorylation by PKA (see below).

In accordance with the observed consensus sites for PKC-phosphorylation on CFTR [3], phosphorylation of CFTR by PKC was observed. The effects of PKC-phosphorylation have been less investigated; it seems to stimulate the response to PKA [30]. A recent report shows that phosphorylation by basal PKC-activity is required for activation by PKA [31]. cGMP-dependent protein kinases (PKG or

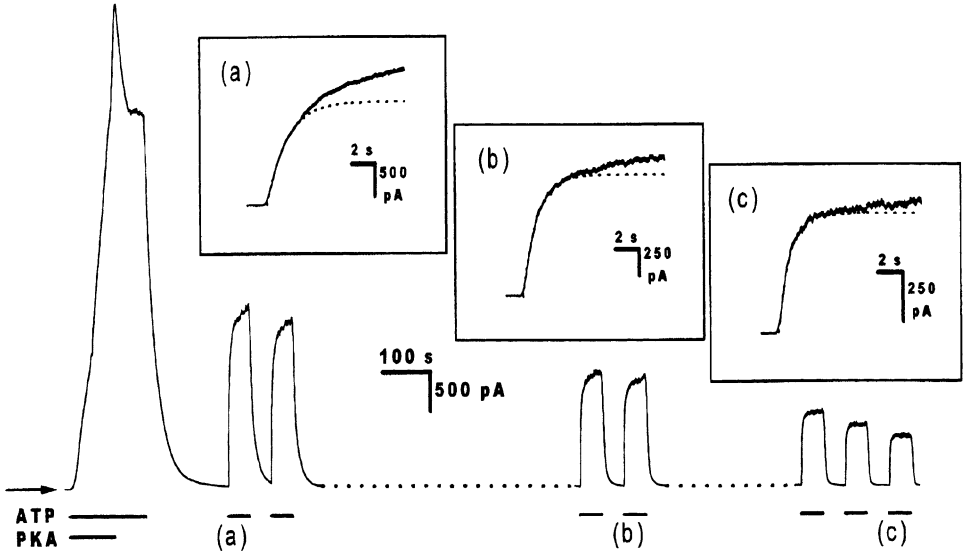

Fig. 2. Fast and slow 'rundown' of steady-state CFTR current, induced by removal of PKA activation. Insets show change of relaxation constants concomitant with rundown. Reproduced from Weinreich et al. [43], with copyright permission of the Rockefeller University Press.

cGK) were also shown to phosphorylate CFTR, and cGK II is able to activate CFTR which might be important physiologically in intestine where cGK II is predominantly expressed [32].

3. Hydrolyzable nucleoside triphosphates are required to open CFTR

Phosphorylation by PKA is a necessary, however not sufficient, requirement to open CFTR channels. As shown in Fig. 1, the chloride current decays to zero upon removal of ATP. The same dependency of CFTR upon ATP was shown for heterologously expressed CFTR in different expression systems [33,34]. By using the specific PKA-inhibitor PKI it could be shown that the action of ATP is completely independent of the ATP-requiring activity of PKA [27,34].

It was then shown that other nucleoside triphosphates could replace ATP, albeit with less potency, whereas ADP or the non-hydrolyzable ATP-analog AMP-PNP were unable to open CFTR channels. This was interpreted as indication that ATP hydrolysis is involved in CFTR gating [27,34]. Further support for the hypothesis that ATP hydrolysis occurs during CFTR gating came from the observation that Mg^{2+} is another necessary cofactor for opening [34,35] as well as from direct demonstration of ATPase activity of purified CFTR [36].

The ATP-dependence of CFTR-open probability shows saturation with $K_{1/2}$ constants in the range of 25–300 μM (e.g. [28,33,34]). The rather large variation is probably caused by differences in recording conditions, like temperature (37°C or room temperature) and, more importantly, by the different degree of phosphorylation of CFTR [37]. CFTR's activation by ATP was modeled in analogy to ligand-gated channels by some groups [33,38], although this completely ignores the fact (described in detail below) that ATP hydrolysis and not solely binding and dissociation are governing the gating cycle.

Evidence for irreversible steps in the gating cycle (as expected for involvement of ATP hydrolysis) was presented from careful single-channel analysis observing two open states which were not distributed randomly [35]. A high activation energy for channel opening [39] can also be seen as supporting the notion of ATP hydrolysis. A recent study on the gating of CFTR chloride channels by ATP hydrolysis led to a quantitative analysis of a cyclic gating scheme [40].

4. The degree of phosphorylation modulates open probability and kinetic constants

Much information on the regulation of CFTR has been obtained from single-channel studies, either with BLM or with membrane patches (for review see [13,15,41]). CFTR channel gating, i.e. opening and closing of channels, appears usually slow with little voltage dependence. In most studies gating could be described by one or two open-time constants (the slow one in the range of several 100 ms) and two to three closed-time constants (the slowest one again in the range of several 100 ms, depending on [ATP]), probably depending on taking into consideration a short closed-time constant of 2–10 ms.

Most authors agree that the effect of ATP concentration is on the slow closed time constant with increasing [ATP] leading to a decreasing closed time. A recent study, however, also found a stimulating influence of ATP on the open time constant [40]. As even deleting 11 possible phosphorylation sites for PKA on CFTR still led to PKA-mediated activation [42] the exact status of phosphorylation might be very important in determining these constants.

A different approach to study the kinetics of CFTR was introduced by pre-steady-state experiments studying the response of many CFTR channels to fast solution changes [43]. Characteristic relaxation time constants could be obtained (see insets in Fig. 2) when changing the (cytoplasmic) bath solution to one containing ATP and also upon returning to an ATP-free solution. Fig. 2 shows additionally how the ATP-induced chloride current decreases with a fast (in the range of 10 s) and a slow (in the range of hundreds of seconds) time constant after fast removal of PKA. This 'rundown' must be caused by dephosphorylation of CFTR by membrane-bound protein phosphatases [28]. The insets show that with advancing dephosphorylation activation by ATP becomes faster. Similarly upon withdrawal of ATP the inactivation got faster. Unfortunately this modulation of gating by phosphorylation could not yet quantitatively be analyzed on the single-channel lev-

el. A reversibly increased open probability, though, was already described as depending on the presence of PKA [44]. Recently, it was proposed that the ten dibasic PKA consensus sites control the bursting rate (i.e. the inverse of the main closed time) and open probability of CFTR by increasing the apparent affinity for ATP [37]. Additional factors must come into play, however, as in the example of Fig. 2 the $K_{1/2}$ for ATP of partially phosphorylated CFTR is about 80 μM [28,43] and [ATP] is, when applied, 500 μM, i.e. virtually saturating.

5. A non-hydrolyzable ATP-analog binds to CFTR and inhibits ATP-mediated opening

As soon as the nucleoside triphosphate requirement for CFTR opening was recognized the effect of hydrolysis-resistant ATP-analogs was studied. Adenylylimidodiphosphate (AMP-PNP), an ATP-analog that is structurally very similar to ATP, but resistant to hydrolysis by many ATPases, was unable to open PKA-phosphorylated CFTR so that ATP hydrolysis was invoked as requirement for CFTR opening [27,34]. This was disputed by some on the grounds that no inhibitory interaction between AMP-PNP and CFTR in experiments with ATP and AMP-PNP was observed [45,46]. Such inhibition of steady-state current or open-probability of single channels is indeed expected if AMP-PNP-binding to CFTR is competitive with ATP. But this expected inhibition is difficult to observe because of the activating effect of AMP-PNP under certain conditions (see below). Recently, evidence was obtained for inhibitory binding by AMP-PNP from measurements of the dwell time until channels got locked open by AMP-PNP [47] and from pre-steady-state experiments with fast solution changes between AMP-PNP and ATP [43], see Fig. 3.

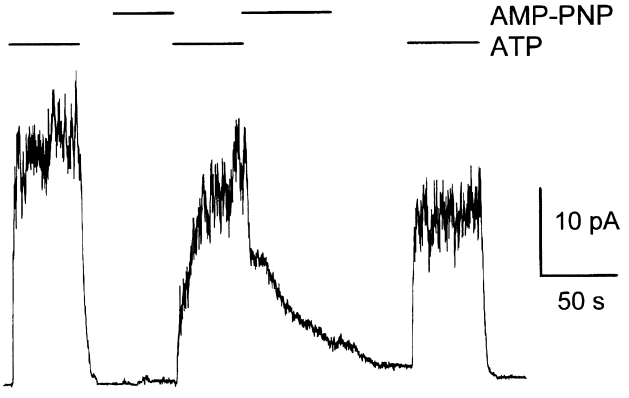

Fig. 3. The effect of ATP and AMP-PNP on PKA-phosphorylated CFTR. Reproduced from Weinreich et al. [43], with copyright permission of the Rockefeller University Press.

Fig. 3 shows that AMP-PNP is not able to open CFTR channels but a subsequent change to ATP reveals by its slowed response that AMP-PNP must have bound to CFTR. The much slower activation by ATP therefore reflects the slow dissociation of AMP-PNP from CFTR.

6. The non-hydrolyzable ATP-analog AMP-PNP reveals the modulating function of a second nucleotide binding site

Fig. 3 also shows that, when changing from ATP to AMP-PNP, a substantial portion of CFTR channels exhibits drastically slowed closing upon removal of ATP (even after removal of AMP-PNP), when compared to the control changes from ATP to zero nucleotide at the beginning and end of the current recording. Such a stimulating action of the non-hydrolyzable ATP-analog AMP-PNP was first observed with human CFTR in sweat gland ducts [48] although it was not recognized at the time that residual ATP was crucial for the observed stimulating action of AMP-PNP. The stimulating effect of AMP-PNP was therefore interpreted as evidence against involvement of ATP hydrolysis in CFTR channel opening [48].

The stimulating effect of AMP-PNP could be explained by binding of AMP-PNP to a second site, as first suggested by Hwang et al. [44] for cardiac CFTR. The authors proposed that binding of AMP-PNP to this site prevents closing until it slowly dissociates from CFTR. But because this site should normally be occupied by ATP and because CFTR channels close much faster upon removal of ATP in the absence of AMP-PNP (see first and last ATP pulse in Fig. 3) it was concluded that ATP also gets hydrolyzed at this second site [44]. These studies led to a new model of CFTR regulation by ATP binding and hydrolysis at two nucleotide binding sites (NBD) which will be outlined below. The involvement of

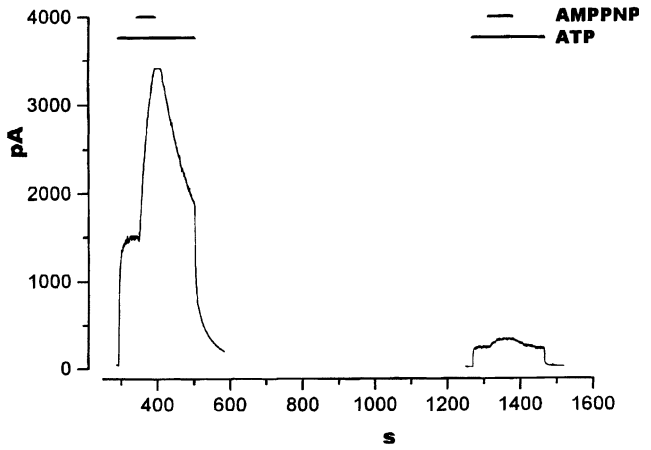

Fig. 4. Phosphorylation-dependent stimulation of CFTR by a mixture of ATP and AMP-PNP. From Weinreich and Nagel, unpublished observations.

two NBD in the regulation of CFTR was then also confirmed for human epithelial CFTR [49], heterologously expressed and studied in BLM.

Applying a mixture of ATP and AMP-PNP should therefore result in an increase of the open probability of CFTR. This was indeed observed with the appearance of channels with greatly prolonged open times, referred to as 'locked open' states [44]. It was also observed that the probability of channels to become locked in the open state depends on their degree of phosphorylation by PKA [44]. The increase of open probability, and therefore chloride current, by a mixture of ATP and AMP-PNP is demonstrated in Fig. 4 for a membrane patch containing ten thousands of CFTR channels. It is evident that the stimulating effect of AMP-PNP is stronger in the beginning of the experiment, shortly after exposure to PKA, compared to the end when substantial rundown (dephosphorylation) has occurred.

In the study by Hwang et al. [44], CFTR conductance was also measured with the whole-cell patch-clamp technique whereby intracellular nucleotide concentrations could be modified via patch pipette perfusion. CFTR was studied after selective inhibition of protein phosphatases (1 and 2a) by okadaic acid which led to partially phosphorylated CFTR channels after removing the stimulus for PKA activation. Whereas the conductance of highly phosphorylated CFTR (i.e. during PKA-activation) was enhanced by a mixture of ATP and AMP-PNP, partially phosphorylated CFTR showed no response to such a mixture [44]. This behavior is very similar to the weak effect of AMP-PNP long after PKA-activation, as shown in Fig. 4.

From these observations a model of CFTR regulation by incremental PKA-mediated phosphorylation and differential binding of ATP to two functionally different sites was set forth [44], see Fig. 5. The model can be described by the following restraints:

1. CFTR has to be phosphorylated on at least one site to yield partially phosphorylated CFTR. These channels can be recognized by their low open probability (P_o) in single-channel measurements and are called partially phosphorylated CFTR.
2. ATP binding and hydrolysis at one nucleotide binding site (which was called NBD-A) leads to opening (bursts) of partially phosphorylated CFTR. Channels will close permanently after dissociation of the hydrolysis products.
3. If CFTR is phosphorylated at one or more further site(s) ATP binds at NBD-A and then another ATP can bind to a second site (NBD-B) where it prolongs the openings of CFTR, most probably by inhibiting release of hydrolysis products at NBD-A.
4. If AMP-PNP, instead of ATP, binds to NBD-B, the channels stay open for very long times (they become 'locked' open). Therefore it can be concluded that normally ATP also gets hydrolyzed at NBD-B because much faster closing is observed after ATP removal when compared to AMP-PNP removal.

In summary, this model proposes ATP hydrolysis at one site (NBD-A) to be required for CFTR opening and optional ATP hydrolysis (or very slow dissociation as in the case of AMP-PNP) at another site (NBD-B) to precede CFTR closing. Therefore this model implies no absolute requirement for ATP hydrolysis at NBD-B to close CFTR. Specifically, it hypothesises that partially phosphorylated (low-P_o-CFTR) channels do not allow binding of ATP to NBD-B which leads to relatively fast dissociation of hydrolysis products at NBD-A and therefore faster closing of CFTR.

Keeping in mind the primary structure of CFTR

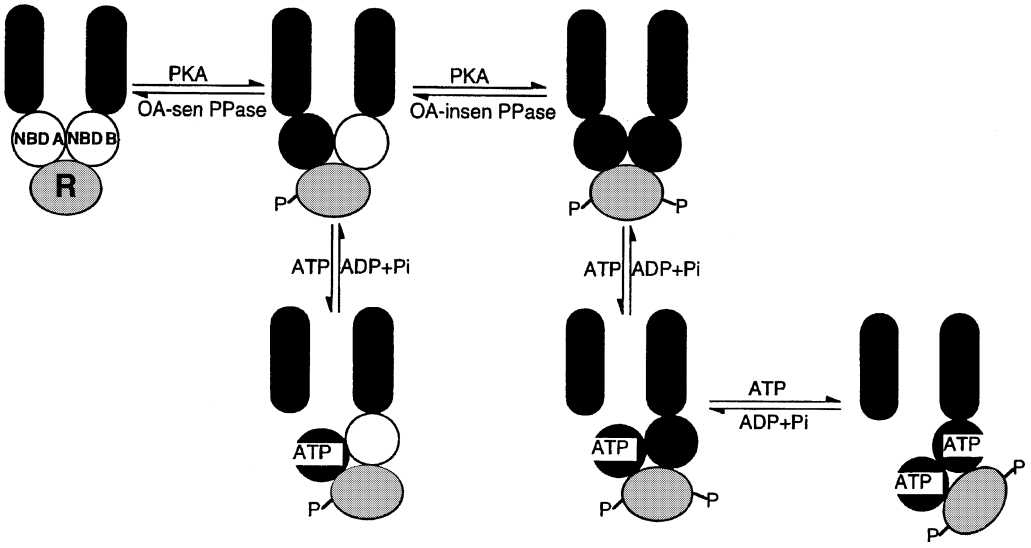

Fig. 5. Model of phosphorylation-dependent action of ATP at two nucleotide binding sites. For details see main text. From Hwang et al. [44], with permission. Copyright (1994) National Academy of Sciences, USA.

with its nucleotide binding folds NBF1 and NBF2 [3], the question emerges which NBF could be NBD-A or NBD-B. From experiments with mutants with defective NBF1 or NBF2 [40,49–51], it was concluded that NBD-A most probably is on NBF1 and NBD-B on NBF2.

It can be assumed that specific phosphorylation sites on CFTR control the accessibility of NBD-A and NBD-B which, however, are not yet identified. The study by Mathews et al. [37] was a first step towards this goal by pointing out the importance, but not exclusivity of the dibasic PKA-sites in control of NBD-B. They found that even 8SA-CFTR (where eight dibasic PKA-consensus-site serines on the R-domain were replaced by alanines) could be locked open by AMP-PNP although with longer delay.

Gunderson and Kopito [35] introduced a different model for CFTR-regulation at two nucleotide binding sites. In their model, ATP hydrolysis at NBF1 'activates' CFTR, but only binding of ATP to NBF2 opens the CFTR channel. Therefore ATP hydrolysis (or dissociation in the case of AMP-PNP) at NBF2 is absolutely required for CFTR to close. Probably they did not find the distinction between partially and highly phosphorylated CFTR because in their system CFTR was always sufficiently phosphorylated to allow binding of ATP or AMP-PNP to NBF2.

7. The modulating effect of ATP hydrolysis products and their analogs

It was shown earlier that ADP is not able to open CFTR chloride channels [27,34]. ADP shows a strong inhibitory effect on CFTR activity when used in mixtures with ATP [45,52]. This inhibitory effect of ADP could be modeled well by competitive binding of ADP instead of ATP with an apparently higher affinity for ADP than for ATP [43,45,52].

The modulating effect of ADP was studied further in pre-steady-state experiments with fast solution change. Fig. 6 shows the CFTR chloride current in response to the indicated nucleotides in A and at higher time resolution in B. Weinreich et al. [43] found a slowed relaxation constant when changing from an ADP- to an ATP-containing solution, as expected for competitive binding of ADP to NBD-A (= NBF1), see Fig. 6B, left column: trace b compared to a or c. Rather unexpected and surprising, however, was the finding that ADP accelerated the closing of CFTR when changing from an ATP- to an ADP-containing solution (Fig. 6B, right column: trace b compared to a or c) [43]. Because this observation could not be explained by competitive binding of ADP to NBD-A (= NBF1) we hypothesized binding of ADP to another site, either binding to NBD-A with still bound P_i or binding to NBD-B (= NBF2).

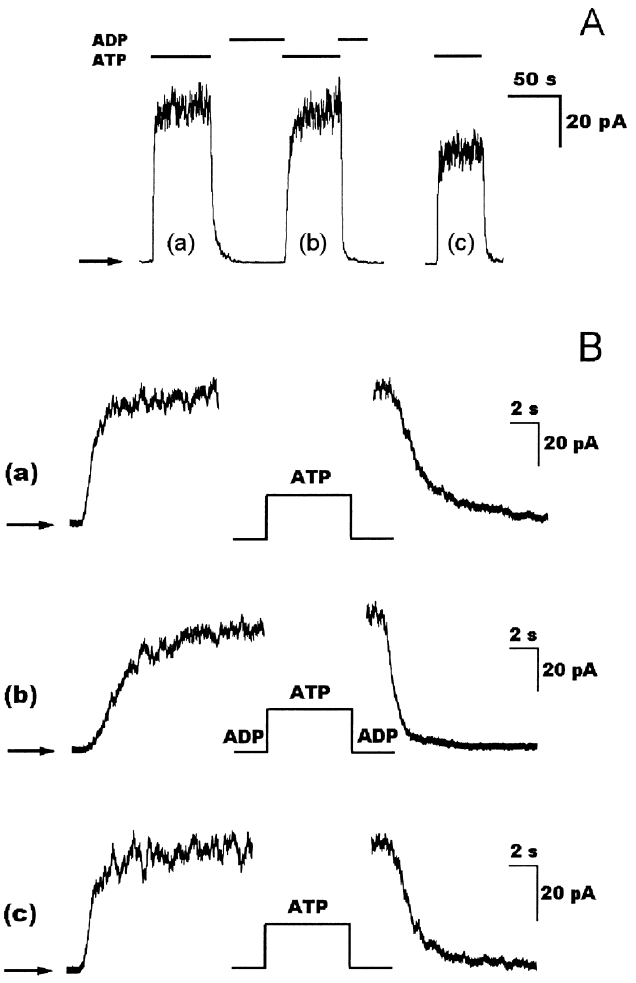

Fig. 6. Modulation of relaxation constants by ADP. For details see main text. Reproduced from Weinreich et al. [43], with copyright permission of the Rockefeller University Press.

In principle, acceleration of CFTR closing by reversal of ATP hydrolysis via ADP and CFTR-bound P_i should be possible if after ATP hydrolysis ADP is released first. However, this is unlikely to be the case for another member of the ABC transporter family, MDR, where substantial evidence was accumulated for a mechanism with P_i release preceding ADP release (for review see [53]). We therefore turned to a model where ADP-binding to NBF2 accelerates closing of CFTR, see Section 8 and Fig. 7.

When studying mutants in NBF1 and NBF2, Anderson and Welsh [52] concluded that the inhibitory effect of ADP is mediated at NBF2. Mutants in NBF2 showed no inhibition by ADP. However, the (to ATP) competitive inhibition of CFTR opening by ADP would be expected to occur at NBF1. It remains therefore the possibility that ADP-binding to NBF2 and ATP-binding to NBF1 are antagonistic, leading to competitive behavior, even if not competing for binding to the same site. This could be analogous to the recent finding for SUR1, another ABC 'transporter', that MgADP, through binding at NBF2, antagonises binding of ATP at NBF1 [54]. Certainly more experiments are necessary to decide this open question.

Inorganic phosphate, the other hydrolysis product, could be expected to inhibit, similarly to ADP. Surprisingly, this is not the case [55]. Phosphate-stimulated CFTR chloride channels, with an estimated EC_{50} of 5 mM, by increasing the open probability. An effect of phosphate on single-channel conductance could be excluded. Single-channel analysis concluded that "the primary effect of P_i was to increase the rate of channel transition to the burst (open) state, with negligible effects on the bursts themselves" [55]. Although most likely the effect of P_i is mediated via NBF1 or NBF2, at present the site of action is not clear.

Earlier it was found that the phosphate transition state analogs ortho-vanadate or beryllium fluoride also have a strong stimulatory influence on CFTR,

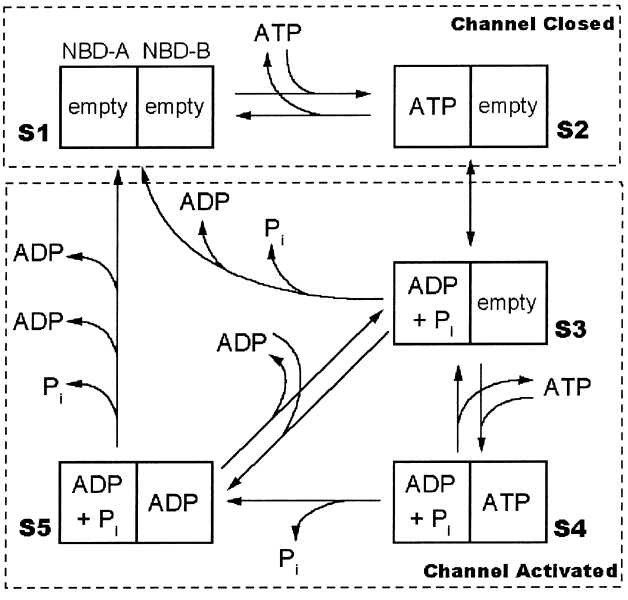

Fig. 7. Model of CFTR regulation by interaction of nucleotides at two NBD. Reproduced from Weinreich et al. [43], with copyright permission of the Rockefeller University Press.

but in contrast to inorganic phosphate, by locking the channel in the open bursting state [56]. In fact, mean burst times were increased by two to three orders of magnitude [49,56]. These effects were interpreted as additional evidence for coupling of CFTR gating to an ATP hydrolysis cycle. As for inorganic phosphate, it is not clear, from these studies, at which NBF vanadate and other phosphate analogs exert their effect. A recent biochemical study with CFTR containing membrane fragments [57] showed that ortho-vanadate enhanced nucleotide occlusion at NBF1 and induced nucleotide occlusion (not seen without ortho-vanadate) at NBF2. A possible conclusion, therefore, is that vanadate acts at NBF1 which would be in agreement with the observation that the degree of phosphorylation is not crucial for the effect of vanadate [56]. Ma et al. found [58] that a mutant, lacking most of the R-domain (aa 708–835), shows inhibition by ortho-vanadate. Further experiments are needed to clarify vanadate's site of action.

A similar drastic increase of open burst time was also described for pyrophosphate (PP_i) [49], again without knowing the site of action. Carson et al. [50] speculated that PP_i interacts with NBF2 because mutations of conserved residues in NBF2, but not in NBF1, significantly reduced the stimulatory effect of PP_i on CFTR [15]. The above-mentioned mutant, lacking most of the R-domain, showed inhibition by PP_i whereas CFTR-WT was stimulated by PP_i at concentrations up to 5 mM and increasingly inhibited at higher concentrations [58] which could indicate interaction of PP_i at two different sites.

8. A model for CFTR regulation by ATP and ADP

Investigation of CFTR chloride channel gating has uncovered a complex mechanism for the regulation of channel opening and closing by protein phosphorylation and interaction with different nucleotides. Although it is quite clear that the degree of phosphorylation modulates activity, so far there exist only vague ideas how this is accomplished. From studies with mutants it became clear that higher phosphorylation increases the apparent affinity to ATP [15,37]. But phosphorylation must also increase the open probability at saturating ATP concentration as can be deduced from the strong stimulating effect of PKA, seen in Fig. 2 where [ATP] is very likely close to saturation [28,43] throughout the shown time of recording.

Several models for CFTR gating by nucleotides have been proposed over the last 5 years. These include models with ATP binding and dissociation, analogous to ligand-gated channels, e.g. simulating the ATP- and ADP-dependence of CFTR-open probability quite well [33,38,45], and models involving ATP hydrolysis at one or two sites ([15,40, 43,44,50,53]) which explain much better the effects observed with nucleotide- and hydrolysis product-analogs.

When analyzing ATP-dependent single-channel gating of CFTR it is tempting to assume that the observed channel dwell times correspond (inversely) to rate constants of the gating cycle. At least two serious problems make an assignment difficult: (1) the (unknown) degree of phosphorylation will modulate the determined constants; and (2) whereas obviously for CFTR gating, more than two intermediates of CFTR exist, only two (or with a substate three) states, i.e. an open and a closed state, can be distinguished in single-channel analysis. Analysis of dwell times often yields several time constants as this was also the case for CFTR. But it is not obvious how to calculate the 'turnover time' for CFTR gating from these constants. In most studies, the sum of open and closed times amounts to less than 1 s, whereas pre-steady-state relaxations yield constants of $\sim$1 s and longer (depending on degree of phosphorylation) [43]. It also has to be kept in mind that even for energy-driven cyclic gating schemes, reversible partial reactions are possible and are in fact also observed for active ion-motive ATPases, like, for example, the Na^+,K^+-ATPase (for review see [59]). In the case of CFTR, it is therefore conceivable to observe reversible openings and closings despite an irreversible gating cycle.

An alternative approach to steady-state measurements of single-channel gating are pre-steady-state experiments with fast solution changes and measuring the relaxation of many channels to a new steady state. This approach is widely used for the investigation of active transport systems with their much smaller current contribution by a single molecule (for review see [59]). It showed for CFTR that during

the lifetime of a typical patch clamp experiment considerable changes of relaxation time constants can be observed [43]. Generally the rates for activation and inactivation become faster with continuing dephosphorylation. This could be caused by changes in the availability of NBD-B (i.e. preferring the S3 to S1 transition at lower phosphorylation, see Fig. 7) and/or by changes in individual rate constants or equilibriums.

The model of CFTR regulation by Hwang et al. [44], shown in Fig. 5, was further developed by Senior and Gadsby [53] and Gadsby and Nairn [13]. Based on functional and sequence similarity to G-proteins, it was proposed that NBF2 always binds a nucleotide, either ADP or ATP. Such a model can accommodate the slowed opening of CFTR when changing from ADP to ATP, but it cannot explain the accelerated closing, observed when changing from ATP to ADP, see Fig. 6. Therefore we adapted the model to take account of the observed effect of ADP, see Fig. 7 [43].

The model of CFTR regulation by nucleotides, shown in Fig. 7, is a minimal model consisting of two closed and three activated states of CFTR. The activated state was introduced instead of an (in the literature more popular) open state to define a state which could be an open state or a state that actually embodies an equilibrium between a closed and an open state (e.g. open burst). This seemed necessary as long as kinetic constants from steady-state and pre-steady-state experiments were not carefully compared for consistency, even though it is quite conceivable and might turn out to be true that the activated state is in fact an open state.

In contrast to previous models [13,15], the closed states S1 and S2 show no bound nucleotide on NBD-B (NBF2), basically because no evidence for such tight ADP-binding in pre-steady-state experiments was found, whereas the slowed opening in experiments with solution changes from ADP to ATP (see Fig. 6) hints at [ADP]-dependent binding of ADP to state S1 (possibly at NBD-A, but see [52]). The activated state S3 must have an empty NBD-B site as can be deduced from the accelerated closing in the presence of ADP. This accelerated closing by ADP is explained by binding of ADP to NBD-B at S3 which leads to state S5 and fast closing.

If ATP instead of ADP binds to NBD-B on S3 this will lead to a prolonged activated state S4 which requires ATP hydrolysis at NBD-B to reach state S5. The ADP then bound to NBD-B induces fast conversion to the closed state S1. Also in this model, ATP binding to NBD-B (at S3) is limited by the degree of phosphorylation of CFTR [44]. Whether ADP binding to NBD-B at S3 is also limited by the degree of phosphorylation, cannot be answered for certain as yet. The single relaxation constant obtained when changing from ATP to ADP (see Fig. 6 and [43]) seems to suggest otherwise. Obviously more experiments are needed to answer such specific questions.

In summary, this model can explain quite well the observed interaction of nucleotides with CFTR. But kinetic constants in this cyclic model are dependent on the degree of phosphorylation of CFTR (see Fig. 2). To sort out the contribution to this cycle of more than 11 phosphorylation sites by PKA alone, most of them activating, some even inhibiting [60], will certainly take a heroic effort. It can at least be begun by the choice of mutants which should show less variability [37].

9. Implications for other ABC transporters?

CFTR is a channel which, once open, passes enough current to study the activity of a single CFTR molecule. This feature clearly discerns CFTR from other ABC transporters, with the exception of SUR, the regulator of a potassium channel [61], and makes it attractive as a model for the interaction of ABC transporters with nucleotides. Although single-channel experiments are powerful, they often need long recording times and recordings from many channels to obtain statistically significant data. Being steady-state experiments, they still can miss some information. Studying thousands of CFTR molecules with a pre-steady-state technique [43,51,60], more common to the study of active transporters [59], provided additional information.

As the ABC transporter family is huge, with members from microorganisms to mammalians, also the structural organization of these proteins is quite diverse. For example, many microorganisms 'construct' an ABC transporter from separate polypeptides and often the two nucleotide binding domains

are built by the same polypeptide. With such an architecture, we should not expect to see the same diversity in function for the two NBD as we have seen for CFTR. On the other hand, the ability of both NBD of CFTR to hydrolyze ATP was conserved in evolution which might indicate more common design features than expected from the different functions of CFTR and most ABC transporters.

Common principles for ABC transporters probably exist and a comparison of the three-dimensional structure of the NBD will be especially interesting, now that the first NBD structures from microorganisms are known, see [62], and more are soon to come. Functional similarities were indeed found for CFTR and P-glycoprotein (Pgp) [53], but also glaring differences which could be expected considering that they evolved to different functions. Mutating the second NBF in Pgp, for example, completely inhibits its pumping function [53] whereas the corresponding mutation leads to prolonged openings of CFTR channels [35,40,50,51]. Recent biochemical measurements on the above mentioned NBF2 mutant of CFTR showed that its ATPase activity was inhibited significantly, by greater than 50% [63]. Surprisingly the corresponding NBF1 mutant of CFTR, its ATPase activity again inhibited significantly, showed no significant inhibition of the rate of channel gating. From these results it was suggested that ATPase activity might not be tightly coupled to channel gating [63]. My personal view would suggest that these observations reinforce the need for cautious interpretation of single-channel data. Clearly, more experiments, if possible by using completely different approaches, are needed to resolve these exciting questions.

Acknowledgements

I am grateful to Frank Weinreich for carefully planning and executing experiments, to Chang Hwang, David Gadsby, Jack Riordan, and Klaus Fendler for fruitful discussions, and to Ernst Bamberg for support and constant encouragement. Supported by the Max-Planck-Gesellschaft and the Deutsche Forschungsgemeinschaft.

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