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Conformational change opening the CFTR chloride channel pore coupled to ATP-dependent gating

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ABSTRACT

Opening and closing of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel are controlled by ATP binding and hydrolysis by its nucleotide binding domains (NBDs). This is presumed to control opening of a single "gate" within the permeation pathway, however, the location of such a gate has not been described. We used patch clamp recording to monitor access of cytosolic cysteine reactive reagents to cysteines introduced into different transmembrane (TM) regions in a cysteine-less form of CFTR. The rate of modification of Q98C (TM1) and I344C (TM6) by both [2-sulfonatoethyl] methanethiosulfonate (MTSES) and permeant $Au(CN)_2^-$ ions was reduced when ATP concentration was reduced from 1 mM to 10 μM, and modification by MTSES was accelerated when 2 mM pyrophosphate was applied to prevent channel closure. Modification of K95C (TM1) and V345C (TM6) was not affected by these manoeuvres. We also manipulated gating by introducing the mutations K464A (in NBD1) and E1371Q (in NBD2). The rate of modification of Q98C and I344C by both MTSES and $Au(CN)_2^-$ was decreased by K464A and increased by E1371Q, whereas modification of K95C and V345C was not affected. These results suggest that access from the cytoplasm to K95 and V345 is similar in open and closed channels. In contrast, modifying ATP-dependent channel gating alters access to Q98 and I344, located further into the pore. We propose that ATP-dependent gating of CFTR is associated with the opening and closing of a gate within the permeation pathway at the level of these pore-lining amino acids.

1. Introduction

Cystic fibrosis is caused by genetic mutations that lead to loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial cell Cl^- channel [1]. CFTR is a member of a large group of membrane ATPase proteins, the ATP binding cassette (ABC) transporter family, the vast majority of which act as active transporters that directly couple ATP hydrolysis to substrate transport across the cell membrane [2]. CFTR however acts not as an active transporter but as an ion channel in which ATP binding and hydrolysis lead to the opening of a pore that mediates the rapid electrogenic transport of Cl^- and other small anions [3,4].

CFTR and other ABC proteins share a modular architecture consisting of two transmembrane domains (TMDs) of six membrane-spanning α-helices each, and two cytoplasmic nucleotide binding domains (NBDs). These two homologous halves are joined by a unique cytoplasmic regulatory domain (R domain), phosphorylation of which is an absolute pre-requisite for channel activation. In all ABC proteins, the NBDs are the site of ATP binding and hydrolysis, which then leads to conformational changes in the TMDs that allow substrate transport [2,5,6]. In CFTR, the TMDs can exist in either "open" or "closed" channel conformations, however, the structural differences between these two conformations are not known. It has been suggested that in ABC proteins that act as active transporters, as with all membrane pumps, multiple "gates" must exist within the substrate translocation pathway, such that this pathway is never continuously open and thus preventing passive substrate movement [3,4]. In contrast, as an ion channel it is supposed that opening and closing of CFTR reflects the activity of a single functional gate, and that opening and closing of this gate is directly controlled by the NBDs in channels that have already been activated by PKA-dependent phosphorylation [4]. This has led to the description of CFTR as a "broken pump" which ultimately evolved from an active transporter ancestor due to atrophy or uncoupling of one of its gates [3,4].

The mechanism by which CFTR gating is controlled by a cyclic scheme of ATP binding and hydrolysis at the NBDs has been well studied [7–9]. Briefly, in channels that have been activated by protein kinase A (PKA)-dependent phosphorylation of the R domain, it is

Abbreviations: ABC, ATP-binding cassette; BHK, baby hamster kidney; CFTR, cystic fibrosis transmembrane conductance regulator; MTS, methanethiosulfonate; MTSES, [2-sulfonatoethyl] methanethiosulfonate; NBD, nucleotide binding domain; PKA, protein kinase A; PPi, pyrophosphate; TM, transmembrane α-helix; TMD, transmembrane domain

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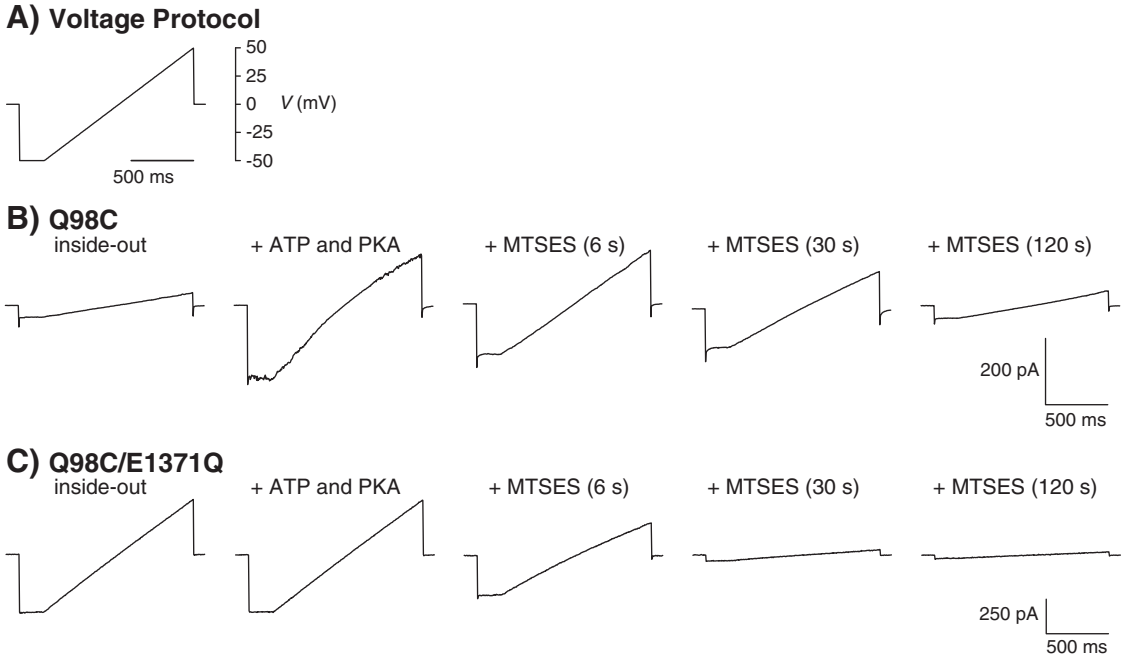


Fig. 1. Examples of currents in inside-out membrane patches during voltage ramps. (A) Depolarizing voltage ramp protocol used. This voltage ramp was applied every 6 s from a holding membrane potential of 0 mV. (B) Raw currents carried by Q98C following patch excision from the cell (inside-out), following channel activation with ATP (1 mM) and PKA, and at different times after application of MTSES (200 μM) to the cytoplasmic face of the membrane. (C) Raw currents carried by Q98C/E1371Q under these same conditions. Note that background (leak) currents are small in Q98C prior to addition of ATP and PKA, whereas in Q98C/E1371Q, as with all constructs bearing the E1371Q mutation, large constitutive currents are observed and are not further increased in amplitude by PKA and ATP (see also Refs. [23,24]).

thought that binding of two molecules of ATP causes dimerization of the two NBDs, triggering channel opening. Hydrolysis of one of these ATP molecules may then cause channel closing, followed by the release of hydrolysis products and at least partial dimer dissociation. These ATP-driven events at the NBDs are presumed to be transmitted to the TMDs to control pore opening and closing. Since gating is an ATP-dependent process, the overall channel open probability—which presumably reflects the probability that the gate in the pore is in the permissive conformation—is affected by factors which impact NBD function, such as ATP concentration, nucleotide analogs that prevent channel closure, and mutations of key NBD residues controlling ATP binding affinity and hydrolysis rate.

While the NBDs are presumed to control a single functional gate in the pore, the proposed structural rearrangements in the TMDs that are controlled by the NBDs to open and close the channel have not been described. Specifically, while localized changes in pore structure have been suggested to occur during channel activation and gating [10–14], the location of the putative single ATP-dependent "gate" is as yet unknown. Recently we showed that access of cysteine-reactive methanethiosulfonate (MTS) reagents from the cytoplasm to parts of the CFTR channel pore was prevented in channels that had not been activated by PKA, but that access to these same sites was rapid in activated channels [14–16]. There appears to be a sharp boundary between residues that could be modified prior to channel activation (located close to the cytoplasmic ends of the transmembrane (TM) α-helices) and those that required channel activation for modification (located further along the TMs from the inside), suggesting that a physically distinct "barrier" restricts access from the cytoplasm to the narrowest region of the CFTR channel pore in non-activated channels [14,15]. Furthermore, the proximity of pore-lining side chains from different TMs around this "barrier" has been demonstrated by cysteine cross-linking [15]. However, the relationship of this "barrier" in non-activated channels to the ATP-dependent channel "gate" that is responsible for the dynamic opening and closing of phosphorylated, activated channels has not been investigated. Furthermore, while this barrier restricts access to large MTS reagents, its ability to prevent movement of small substances such as Cl^- ions is also unknown.

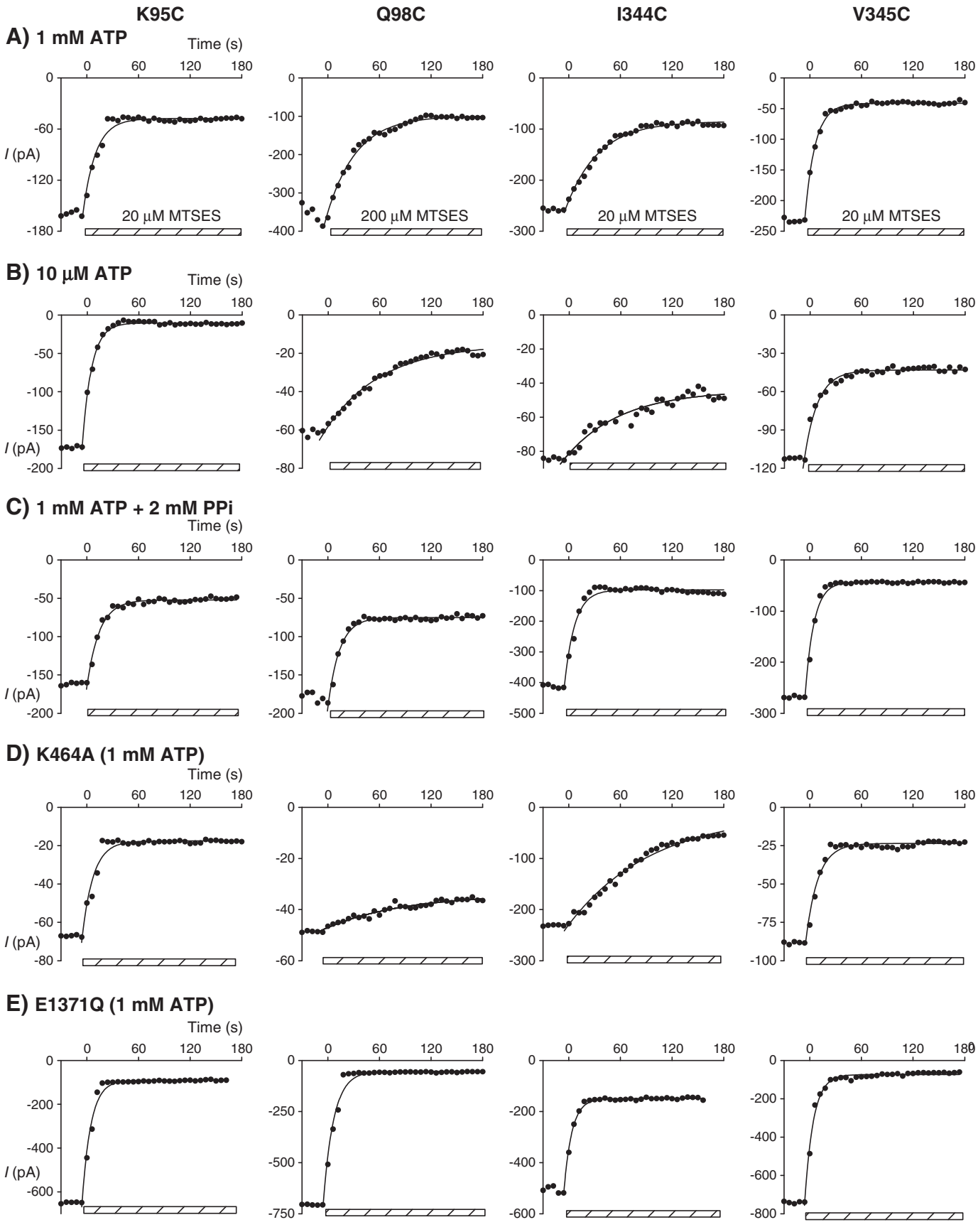
In the present work we have investigated ATP-dependent access of different cytoplasmic substances to different sites in the channel pore, in channels that have already been activated by PKA-dependent phosphorylation. Our work is based on the hypothesis that, for residues beyond the ATP-dependent gate, the rate of access should be altered by changes in NBD function, whereas between the gate and the cytoplasm there should be no influence of gating on the rate of access. We have used both pharmacological and mutagenic manipulation of NBD function to influence overall channel open probability and, presumably, the orientation of the channel gate controlled by the NBDs. Our present results allow us to propose a physical location within the pore for the channel's ATP-dependent gate that regulates flow of Cl^- and other small ions.

2. Materials and methods

Experiments were carried out on baby hamster kidney (BHK) cells transiently transfected with different forms of human CFTR, as described previously [17]. Both wild type CFTR and a variant in which all 18 endogenous cysteine residues have been substituted by other

Fig. 2. Timecourse of modification by MTSES. Example timecourses of macroscopic currents (measured at −50 mV during brief voltage excursions from a holding potential of 0 mV) carried by K95C, Q98C, I344C and V345C as indicated, in inside-out membrane patches. Current amplitudes were measured every 6 s following attainment of stable current amplitude after channel activation. Channels were activated with PKA (20 nM) and either a high concentration of ATP (1 mM; in (A) and (C)–(E)) or a low concentration of ATP (10 μM; (B)). In (C), patches were also treated with PPi (2 mM) following activation by PKA and ATP. In (D) channels also had the NBD1 mutation K464A, and in (E) channels also had the NBD2 mutation E1371Q. In each panel, MTSES (20 μM for K95C, I344C and V345C, and 200 μM for Q98C; see Materials and methods) was applied to the cytoplasmic face of the patch at time zero (as indicated by the hatched bar at the bottom of each panel). The decline in current amplitude following MTSES application has been fitted by a single exponential function in each case.

amino acids ("cys-less" CFTR) [18] were used. The cys-less version used in the present study also includes a mutation in NBD1 (V510A) to increase protein expression in the cell membrane [19]. This cys-less variant has channel pore properties very similar to those of wild type CFTR [20]. Additional mutations were introduced into the cys-less background using the QuikChange site-directed mutagenesis



system (Agilent Technologies, Santa Clara, CA, USA) and verified by DNA sequencing.

Macroscopic CFTR currents were recorded using patch-clamp recording from inside-out membrane patches excised from BHK cells as described in detail previously [21,22]. Following patch excision and recording of background currents (where appropriate), CFTR channels were activated by exposure to PKA catalytic subunit (20 nM) plus MgATP (10 μM–1 mM) in the cytoplasmic solution. In some experiments, CFTR channels bearing the NBD2 mutation E1371Q were used. This mutation results in constitutive, high levels of activity when expressed in BHK cells [23,24] (see Fig. 1C). Both intracellular (bath) and extracellular (pipette) solutions contained 150 mM NaCl, 2 mM $MgCl_2$, and 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (pH adjusted to 7.4 using NaOH). Currents were activated using a high (1 mM) or low (10 μM) concentration of ATP to yield differing levels of overall channel activity. The concentration of ATP required for half-maximal open probability of CFTR is ~50 μM [25], with activity near maximal at 1 mM ATP. In some cases, CFTR channel activity was further increased by addition of 2 mM sodium pyrophosphate (PPi) following activation with 1 mM ATP.

Channels were exposed to intracellular cysteine-reactive reagents to modify introduced cysteine side chains. [2-sulfonatoethyl] methanethiosulfonate (MTSES) was initially prepared as a high concentration (160 mM) stock solutions in distilled water and stored frozen in small volume aliquots until the time of use, when it was diluted in bath solution and used immediately. Potassium dicyanoaurate ($KAu(CN)_2$) was prepared as a high concentration (100 mM) stock in normal bath solution and diluted immediately prior to use.

Following channel activation, CFTR macroscopic current amplitude was monitored during brief voltage deflections to ±50 mV from a holding potential of 0 mV applied every 6 s (Fig. 1). Once macroscopic current amplitude had reached a steady level, channels were exposed to cytoplasmic MTSES or $Au(CN)_2^-$ to modify introduced cysteine side chains [14,15]. In most cases, 20 μM MTSES and 200 nM $Au(CN)_2^-$ were used, however, in channels bearing the Q98C mutation these concentrations were increased to 200 μM MTSES and 200 nM $Au(CN)_2^-$ because of the lower rate of modification of a cysteine at this position ([15], and Figs. 3, 6 and 7). These differences in MTSES modification rate reflect different locations of introduced cysteines within the pore [14–16]. The similar low apparent modification rate for Q98C by $Au(CN)_2^-$ (Figs. 6 and 7) suggests that accessibility of this substance is limited by similar factors as that of MTSES. To measure the rate of modification, the time-dependent change in macroscopic CFTR current amplitude following addition of MTSES or $Au(CN)_2^-$ was fitted by a single exponential function. Since we are interested in monitoring the rate of modification in different channel states, it is possible that the time-dependent change in current amplitude is the sum of multiple exponential components, however, in all cases the small currents obtained appeared well fitted by a single exponential. The measured time constant of exponential current change, τ, was used to calculate the apparent second order reaction rate constant, *k*, from the equation $k = 1/([R]\,\tau)$, where [R] is the concentration of cysteine reactive reagent (MTSES or $Au(CN)_2^-$) applied (see above). As noted above, if there are multiple exponential components then the calculated rate constant will reflect the weighted average of multiple rate constants.

For analysis of the voltage-dependent open channel blocking effects of $Au(CN)_2^-$ (Fig. 5), macroscopic current–voltage (*I–V*) relationships were constructed using depolarizing voltage ramp protocols [21,26]. Background (leak) currents recorded before addition of PKA and ATP have been subtracted digitally, leaving uncontaminated CFTR currents [26,27]. Block was analyzed exactly as described previously for wild type CFTR [22]. Blocker

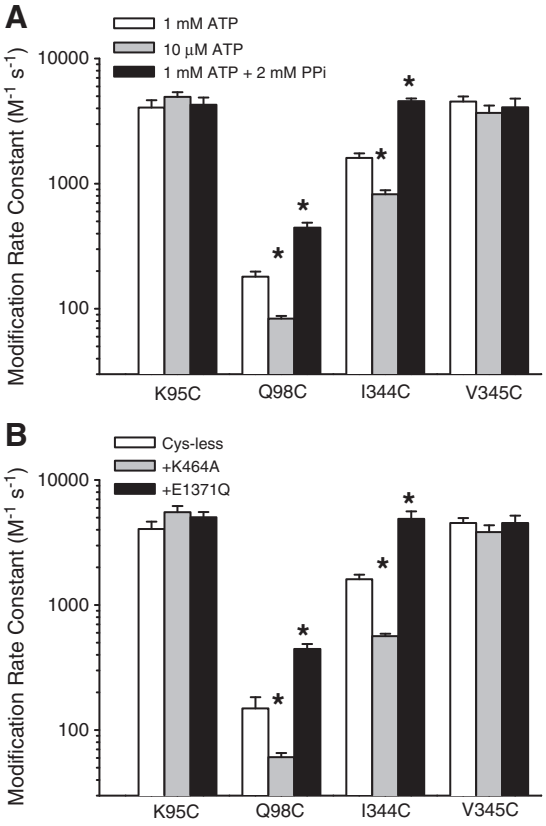


Fig. 3. Effect of altered channel gating on MTSES modification rate constants. Average modification rate constants for MTSES were calculated from fits to data such as those shown in Fig. 2. In (A), NBD function has been altered pharmacologically using different concentrations of ATP and PPi. In (B), NBD function has been altered by the mutations K464A (NBD1) and E1371Q (NBD2). In (A) asterisks indicate a significant difference from PKA and 1 mM ATP, whereas in (B) asterisks indicate a significant difference from cys-less background ($P<0.05$ in each case). Mean of data from 3 patches in each case.

concentration–inhibition relationships (Fig. 5C) were fitted by the equation:

$$\text{fractional unblocked current} = 1/(1 + ([Au(CN)_2^-]/K_d)) \quad (1)$$

where K_d is the apparent blocker dissociation constant.

Experiments were carried out at room temperature, 21–24 °C. Values are presented as mean ± S.E.M. Tests of significance were carried out using a Student's two-tailed *t*-test unless stated otherwise. All chemicals were from Sigma-Aldrich (Oakville, ON, Canada), except for PKA (Promega, Madison, WI, USA) and MTSES (Toronto Research Chemicals, North York, ON, Canada).

3. Results

3.1. Accessibility of cytoplasmic MTSES

Recently we have used cytoplasmically applied MTS reagents to modify cysteine residues introduced into CFTR pore-lining TM1 [15] and TM6 [14]. In these studies, the rate of channel modification by negatively charged MTSES was quantified by measuring the rate of change of macroscopic current amplitude in fully activated channels following MTSES application to the cytoplasmic face of inside-out membrane patches [14,15]. Examples of macroscopic currents recorded from Q98C-CFTR under these conditions are shown in Fig. 1B, and examples of timecourses of MTSES-induced current amplitude change are shown in Fig. 2. As described previously, at K95C and Q98C (TM1) and at I344C and V345C (TM6), current amplitude is decreased by treatment

with MTSES, reflecting modification of these introduced cysteine side chains [14,15]. In Fig. 2A–C, CFTR channel currents have been activated in different ways to yield differing levels of NBD-dependent channel activity (see Materials and methods). Inspection of these example timecourses indicates that, while such manipulations have no effect on the rate of modification in K95C or V345C, the rate of modification is altered in both Q98C and I344C (Fig. 2A–C). Quantification of the mean modification rate constant (as described in Materials and methods) demonstrates that decreasing ATP concentration from 1 mM (Fig. 2A) to 10 μM (Fig. 2B) to decrease channel opening rate significantly decreases the rate of modification in Q98C and I344C (~2.0-fold decrease in modification rate constant; $P<0.01$), whereas the rate of modification of K95C and V345C was apparently unaffected ($P>0.2$) (Fig. 3A). Conversely, treatment with PPi (2 mM; Fig. 2C) to inhibit channel closure and increase open probability significantly increases the rate of modification in Q98C and I344C (2.5–2.8-fold increase in modification rate constant; $P<0.01$) but has no effect on the rate of modification of K95C and V345C ($P>0.4$) (Fig. 3A). These results suggest that pharmacological manipulation of NBD function results in changes in the accessibility of Q98C and I344C—but not K95C or V345C—to cytoplasmic MTSES.

ATP-dependent CFTR channel gating can also be modified by point mutations in the NBDs that alter the rate of ATP hydrolysis. Indeed, previous studies of pore accessibility changes have taken advantage of NBD mutations K464A and E1371Q [10,11]. The NBD1 mutation K464A decreases channel opening rate and has been described as slightly decreasing overall open probability at moderate ATP concentrations [9,28,29], whereas mutagenesis of the key catalytic glutamate residue E1371 in NBD2 prevents ATP hydrolysis to cause dramatic prolongation of CFTR channel open times, presumably leading to an increase in open probability [1,30,31]. To alter channel gating by non-pharmacological means, we therefore combined the NBD mutations K464A and E1371Q with each of the four cysteine mutants described above. Channels were activated using PKA (20 nM) and ATP (1 mM) but not exposed to PPi. Constructs including the E1371Q mutation gave large, spontaneously active currents in BHK cells (Fig. 1C), as described previously, and likely have an open probability close to one [23,24].

Examples of macroscopic current modification of NBD mutant channels by cytoplasmic MTSES are shown in Fig. 2D–E, and the results of these experiments are summarized in Fig. 3B. The K464A mutation significantly decreased the rate of MTSES modification at Q98C and I344C (2.5–2.9-fold decrease in modification rate constant; $P<0.005$) but had no effect on the rate of modification at K95C or V345C ($P>0.5$) (Fig. 3B). Conversely, the E1371Q mutation significantly increased the rate of MTSES modification at Q98C and I344C (3.0–3.1-fold increase in modification rate constant; $P<0.02$) but had no effect on the rate of modification at K95C or V345C ($P>0.25$) (Fig. 3B). These results therefore suggest that altering NBD function non-pharmacologically by mutagenesis alters accessibility of Q98C and I344C to cytoplasmic MTSES, whereas accessibility of K95C and V345C are unaffected by NBD-driven channel gating.

3.2. Cytoplasmic $Au(CN)_2$ effects on wild type CFTR reflect cysteine modification

MTSES is a large molecule that is not permeant in CFTR. As a result, changes in access of MTSES may not be relevant to changes in pore conformation that control the movement of Cl^- ions. Small permeant anions such as $Au(CN)_2^-$ have been used to modify cysteine residues introduced into the CFTR pore [14,32,33]. However, intracellular $Au(CN)_2^-$ also inhibits wild type CFTR by at least two mechanisms [22]. Previously our group separated and characterized these two inhibitory effects as a "gating effect" (a voltage-independent reduction in channel open probability) and a "conductance effect" (a voltage-dependent reduction in the rate of Cl^- flow in open channels [22]). The voltage-independent gating effect of intracellular $Au(CN)_2^-$, isolated by recording at a depolarized voltage of +50 mV, is illustrated in Fig. 4. Interestingly, this inhibitory effect was not observed in cys-less CFTR (Fig. 4). This suggests that the voltage-independent inhibition of CFTR by $Au(CN)_2^-$ (the gating effect) in fact reflects modification of endogenous cysteine residues in the CFTR protein. In support of this idea, inhibition of wild type CFTR by $Au(CN)_2^-$ was not reversed by washing $Au(CN)_2^-$ from the bath but could be reversed by the reducing agent dithiothreitol (2 mM) (data not shown).

Because the voltage-independent gating effect of $Au(CN)_2^-$ on wild type CFTR is not observed in channels that have been treated by PPi [22]—consistent with this effect reflecting modification of channel gating—the overall inhibitory effects of $Au(CN)_2^-$ are strongly PPi-sensitive [22]. In contrast, $Au(CN)_2^-$ inhibition of cys-less CFTR appears identical in the absence or presence of PPi (2 mM) (Fig. 5). At −80 mV, the fitted K_d for $Au(CN)_2^-$ was 84.2 μM in the absence of PPi, and 76.9 μM in the presence of PPi (Fig. 5C); the voltage-dependence of K_d is shown in Fig. 5D. This voltage-dependent blocking effect appears identical to the open-channel blocking "conductance effect" of $Au(CN)_2^-$ previously described for wild type CFTR (following PPi treatment) [22], suggesting that this pore-blocking conductance effect is unaltered in cys-less, and is therefore presumably independent of endogenous cysteine residues in wild type CFTR.

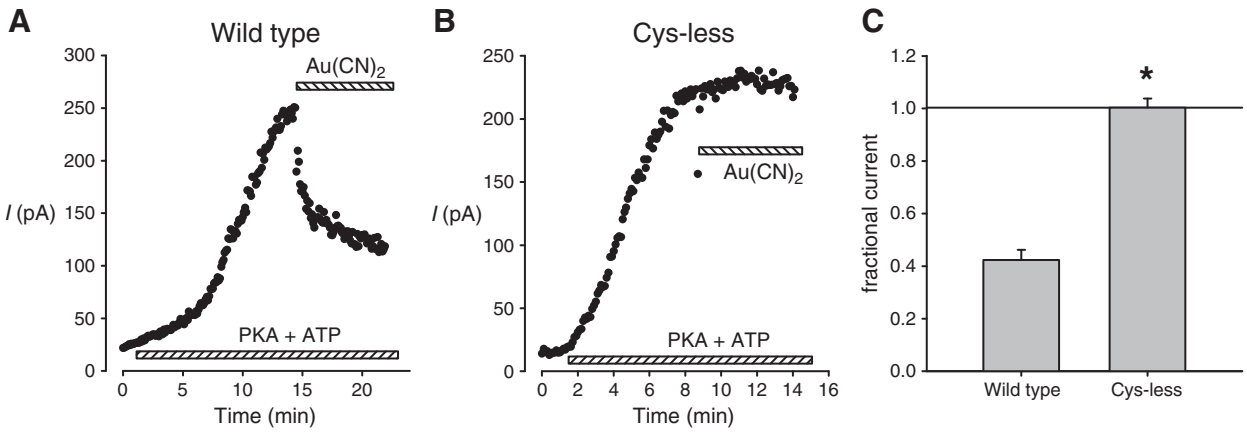


Fig. 4. Inhibition of wild-type CFTR by cytoplasmically applied $Au(CN)_2^-$. (A, B) Example timecourses of macroscopic currents (monitored at +50 mV during brief voltage excursions from a holding potential of 0 mV) for wild type (A) and cys-less (B). Following full current activation with PKA (20 nM) and ATP (1 mM), patches were treated with cytoplasmic $Au(CN)_2^-$ (50 μM). (C) Mean effect of this concentration of $Au(CN)_2^-$ on macroscopic current amplitude. Mean of data from 4 to 5 patches. Asterisk indicates a significant difference from wild type ($P<0.0001$).

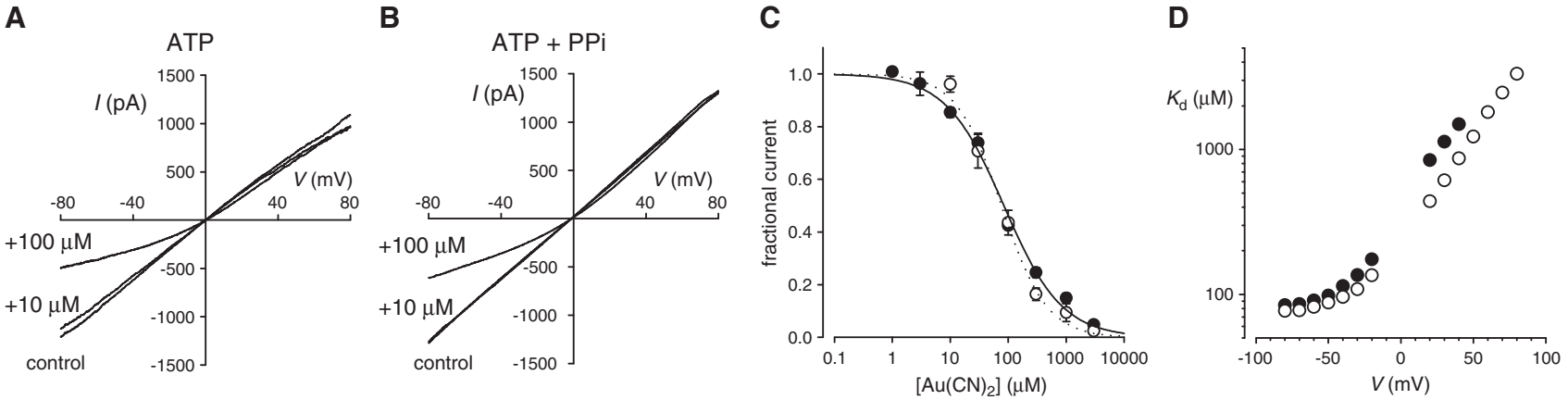


Fig. 5. Voltage-dependent block of cys-less CFTR by cytoplasmically applied $Au(CN)_2^-$. (A, B) Example leak-subtracted *I–V* relationships for cys-less CFTR following activation with PKA (20 nM) plus ATP (1 mM) (A) or ATP (1 mM) followed by PPi (2 mM) (B). In both cases, currents were recorded before (control) and after sequential addition of $Au(CN)_2^-$ to the intracellular solution at the final concentrations indicated. (C) Mean fraction of control macroscopic current amplitude remaining in the presence of different concentrations of $Au(CN)_2^-$ at a membrane potential of −80 mV, in patches in which current was activated by ATP (●) or ATP plus PPi (○). The data have been fitted by Eq. (1), giving a K_d of 84.2 μM in the absence of PPi (●), and 76.9 μM in the presence of PPi (○). Mean of data from 3 to 5 patches. (D) Voltage dependence of K_d estimated from fits such as those shown in (C).

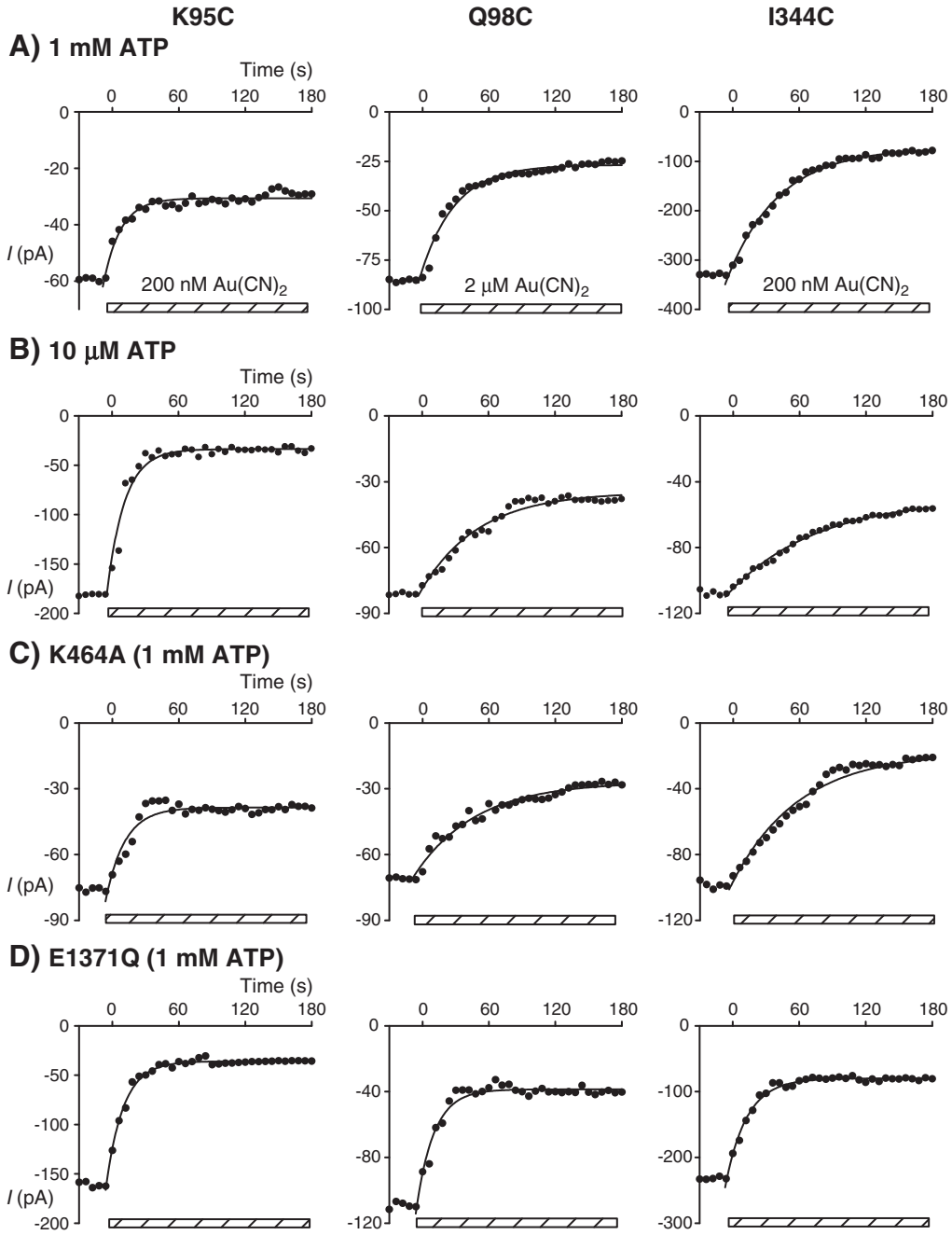


Fig. 6. Timecourse of modification by $Au(CN)_2^-$. Example timecourses of macroscopic currents (measured at −50 mV during brief voltage excursions from a holding potential of 0 mV) in inside-out membrane patches. Current amplitudes were measured every 6 s following attainment of stable current amplitude after channel activation. Channels were activated with PKA (20 nM) and either a high concentration of ATP (1 mM; in (A) and (C)–(D)) or a low concentration of ATP (10 μM; (B)). Reporter cysteines (K95C, Q98C, and I344C as indicated) were examined in isolation (A, B) or combined with the NBD mutations K464A (C) or E1371Q (D). In each panel, $Au(CN)_2^-$ (200 nM for K95C and I344C, and 2 μM for Q98C; see Materials and methods) was applied to the cytoplasmic face of the patch at time zero (as indicated by the hatched bar at the bottom of each panel). The decline in current amplitude following $Au(CN)_2^-$ application has been fitted by a single exponential function in each case.

3.3. Accessibility of cytoplasmic $Au(CN)_2^-$ to introduced cysteines

The above results suggest that cys-less CFTR is an appropriate background in which to study modification of introduced cysteines by $Au(CN)_2^-$ applied cytoplasmically at concentrations <10 μM. In fact, we found that currents carried by K95C, Q98C, and I344C were potently inhibited by much lower concentrations of $Au(CN)_2^-$ (200 nM–2 μM; Fig. 6). Both K95C and I344C were rapidly inhibited by 200 nM $Au(CN)_2^-$ (Fig. 6), reflecting a high modification rate constant (Fig. 7). For Q98C, the modification rate constant was lower, and experiments were carried out using a higher concentration of $Au(CN)_2^-$ (2 μM). Interestingly, the modification rate constant for MTSES is also lower for Q98C (Fig. 3), probably reflecting its location more deeply into the pore [15]. In contrast, V345C was not sensitive to inhibition by low concentrations of $Au(CN)_2^-$, although it appeared to show the same sensitivity as cys-less to the voltage-dependent blocking effects of higher concentrations of $Au(CN)_2^-$ described above (data not shown). We therefore compared the rate of $Au(CN)_2^-$ inhibition in K95C, Q98C and I344C at two different ATP concentrations (10 μM and 1 mM), as well as in channels also bearing the NBD mutations K464A or E1371Q (Fig. 6). Quantification of the mean modification rate constant demonstrated that decreasing ATP

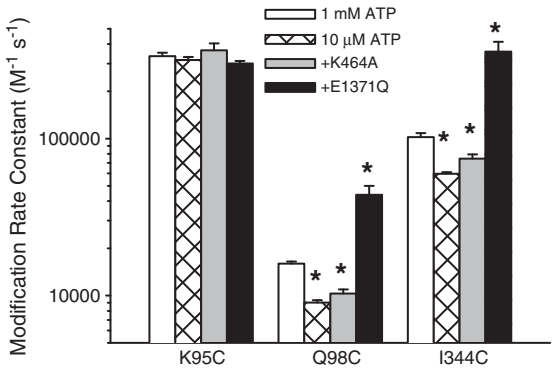


Fig. 7. Effect of altered channel gating on $Au(CN)_2^-$ modification rate constants. Average modification rate constants for $Au(CN)_2^-$ in different channel constructs were calculated from fits to data such as those shown in Fig. 6. Asterisks indicate a significant difference from the same mutant in a cys-less background with 1 mM ATP ($P<0.01$). Mean of data from 3 patches in each case.

concentration to 10 μM significantly decreased the rate of $Au(CN)_2^-$ modification of Q98C and I344C (1.7–1.8-fold decrease in modification rate constant; $P<0.005$) but had no effect on the rate of modification at K95C ($P>0.4$) (Fig. 7). In addition, the K464A mutation significantly decreased the rate of $Au(CN)_2^-$ modification of Q98C and I344C (1.4–1.5-fold decrease in modification rate constant with 1 mM ATP; $P<0.005$) but had no effect on the rate of modification at K95C ($P>0.5$) (Fig. 7). Conversely, the E1371Q mutation significantly increased the rate of $Au(CN)_2^-$ modification at Q98C and I344C (2.8–3.5-fold increase in modification rate constant; $P<0.01$) but had no effect on the rate of modification at K95C ($P>0.2$) (Fig. 7). These results therefore suggest that similar factors within the pore restrict the access of cytoplasmic $Au(CN)_2^-$ and MTSES in an NBD-dependent fashion.

4. Discussion

The function of CFTR as an ATP-gated ion channel suggests that ATP binding and hydrolysis by the NBDs should control the opening and closing of a single functional gate within the permeation pathway. While changes in CFTR activity have been proposed to lead to changes in the conformation of specific parts of the channel pore [10–16], the location of a gate controlling channel opening has not been described. We have used pharmacological and mutagenic manipulations that specifically alter ATP-dependent channel gating to investigate changes in the accessibility of different parts of the pore. All experiments were carried out using channels that had already been activated by PKA-dependent phosphorylation, minimizing the effects of changes in R domain function. For two introduced cysteine residues—K95C in TM1 and V345C in TM6—the rate of modification by cytoplasmic reagents was independent of ATP-dependent channel gating (Figs. 3, 6), suggesting that access to these residues is similar both in open channels and in closed channels. In contrast, nearby residues (Q98C in TM1, I344C in TM6) showed strongly state-dependent accessibility, both to the large MTSES (Fig. 3) and the small, permeant $Au(CN)_2^-$ ion (Fig. 7). The rate of modification at these two sites was significantly decreased by lowering ATP concentration (Figs. 3A, 7) and by the K464A mutation (Figs. 3B, 7), and significantly increased by PPi (Fig. 3A) and the E1371Q mutation (Figs. 3B, 7). Given the well known effects of these manipulations on channel gating and overall open probability, it seems reasonable to suggest that the rate of modification at Q98C and I344C is positively associated with open probability, suggesting that open channels are modified more easily than are closed channels. We therefore propose that the ATP-dependent channel gate that controls the dynamic opening and closing of the pore is located between K95 and Q98 in TM1, and between I344 and V345 in TM6. This location appears equivalent to the "barrier" previously suggested to restrict access in channels prior to PKA-dependent phosphorylation [14,15], suggesting that these previous studies were in fact reporting the effects of increased channel open probability following PKA-dependent phosphorylation. The fact that this putative gate can regulate the movement of permeant $Au(CN)_2^-$ ions into the pore suggests that this gate should also be able to influence the movement of Cl^- ions.

The very high apparent open probability of E1371Q mutant channels in BHK cells [23,24] means that constructs bearing this mutation likely give a good estimate of the rate of modification of open channels. As pointed out above, the lack of apparent state-dependence of modification in K95C and V345C suggests that the rate of modification at these sites is similar in closed channels. However, our measurements of time constants of changes in macroscopic current amplitude in channels that are opening and closing do not allow us to estimate the rate of modification at Q98C and I344C in closed channels. Previously we suggested that the rate of modification of Q98C [15] and I344C [14] is negligible in inactive channels prior to PKA-dependent phosphorylation. It is possible that phosphorylation leads to partial opening of the putative gate and a partial increase in access, and ATP-dependent gating then results in a further increase in access through this region. This could allow slow modification of Q98C and I344C in phosphorylated, but closed, channels. However, a simpler explanation is that the gate prevents access to Q98 and I344 when the channel is closed, and that these residues are modified only in open channels. The measured rate of modification at these sites under different conditions would then be determined both by the rate of modification of open channels and the open probability (which is varied under our different experimental conditions). We have not measured the channel open probability under our experimental conditions, however, previous results suggest that the modification rates we measure are not in fact linearly related to open probability. For example, reducing ATP concentration to 10 μM is expected to decrease open probability around tenfold [25], whereas the K464A mutation is usually reported as having only a minor effect on open probability [9,28,29]. Perhaps surprisingly, then, we find the effects of low ATP and the K464A mutation on modification rate constants for Q98C and I344C to be quantitatively similar and in the range of 1.5 to 3-fold (Figs. 3, 7).

Our present results also provide novel information on the effects of $Au(CN)_2^-$ on wild type CFTR. Cytoplasmic $Au(CN)_2^-$ was originally described as having dual inhibitory effects on CFTR, reducing both open

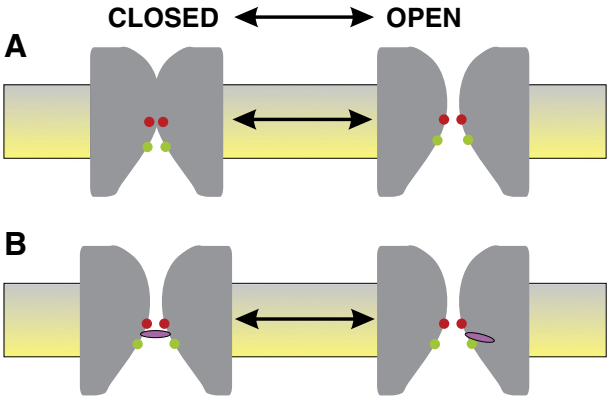


Fig. 8. Simple cartoon models of ATP-dependent gating of the CFTR pore. Both models show the proposed transition of the TMDs between closed and open states, resulting in an increase in accessibility from the cytoplasm to Q98 and I344 located relatively deeply along the axis of the TMs (red) but no change in access to K95 and V345 located more superficially (green). (A) The conformational change (arrow) results in rearrangement of the TMDs (in this case, a physical separation). (B) The conformational change results in removal of a localized physical barrier.

probability (the "gating effect") and Cl^- flux through open channels (the "conductance effect") [22]. While the gating effect appears to be absent in cys-less CFTR (Fig. 4), the conductance effect seems unaltered (Fig. 5). This suggests that the gating effect reflects a reduction in channel open probability that is caused by $Au(CN)_2^-$ modification of endogenous cysteine residues in the CFTR protein. The CFTR protein contains 18 cysteine residues, many of which are in important cytoplasmic domains including NBD1, NBD2, the R domain and the C-terminal tail [34]. Wild type CFTR is also potently inhibited by cytoplasmic MTS reagents [35], an effect which is not observed in cys-less [19], as well as by the cysteine-reactive reagent *p*-chloromercuribenzosulfonate [36]. The endogenous site(s) of modification that presumably underlie these inhibitory effects are not known. Wild type CFTR is reversibly inhibited by glutathionylation of C1344 in NBD2 [37]. In contrast to the cysteine-mediated gating effect, the conductance effect is suggested to reflect open channel block by $Au(CN)_2^-$ ions [17,22] which is independent of cysteine residues in the pore, consistent with earlier single channel recordings of cys-less CFTR [20,32].

While K95C, Q98C and I344C were rapidly inhibited by low concentrations of cytoplasmic $Au(CN)_2^-$ (Fig. 6), V345C showed similar $Au(CN)_2^-$ sensitivity as cys-less CFTR (data not shown). While the reasons why V345C is apparently not modified by $Au(CN)_2^-$ are not clear, we have previously found that not all pore-lining cysteine side chains that can be modified by MTS reagents can also be modified by $Au(CN)_2^-$ [14]. Furthermore, it has been shown that V345C is insensitive to external $Au(CN)_2^-$, although cysteines substituted for other nearby side chains are inhibited under similar conditions [33].

5. Conclusions

Control of CFTR activity is by ATP interactions with the NBDs that trigger a switch between "open" and "closed" conformations of the TMDs. The structural basis of this conformational change is not currently known. We propose that one functional consequence of ATP-dependent channel opening is to allow access of cytoplasmic substances beyond a "gate" located in the inner vestibule of the pore and into the narrow pore region. Residues on the cytoplasmic side of this gate (such as K95 in TM1 and V345 in TM6) are accessible to the cytoplasm in both open and closed channels, whereas residues on the external side of the gate (such as Q98 in TM1 and I344 in TM6) are likely accessible only in open channels. The functional importance of this gate is reflected in the finding that movement of permeant $Au(CN)_2^-$ ions—likely a good surrogate for Cl^-—is regulated at this point in an ATP concentration-dependent manner (Fig. 7). The nature of structural change in the pore associated with opening of the gate is not known. While different structural mechanisms may be used to open ion channels, we envision two different kinds of schemes, that emphasize global (Fig. 8A) or localized (Fig. 8B) changes in pore structure during channel opening. In the global model (Fig. 8A), structural rearrangement of the TMDs lead to dilation and opening of a transmembrane pathway for Cl^- ions. The location of the "gate" then simply reflects the physical limit beyond which cytoplasmic substances cannot pass in closed channels. Such a global rearrangement of the TMDs might be considered consistent with the idea that ABC proteins switch between "inward facing" and "outward facing" TMD conformations during ATP-dependent transport cycles [2,4–6]. In the localized model (Fig. 8B), the overall structure of the permeation pathway is assumed not to change dramatically during opening, but instead removal of a localized barrier at the position of the channel gate allows access through this region. This model has parallels in other ion channel types in which pore opening is thought to be associated with the physical withdrawal of specific amino acid side chains from the permeation pathway [38–41]. These are simplified cartoon models and the actual structural rearrangements underlying channel opening and closing may involve some aspects of both global and local changes, as well as other kinds of structural rearrangements such as rotational movements of individual TMs [13,42]. The role of individual amino acid side chains, such as those located close to the gate, in controlling channel opening and closing remain to be investigated.

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