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Molecular determinants and role of an anion binding site in the external mouth of the CFTR chloride channel pore

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Chloride permeation through the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is blocked by highly lyotropic permeant anions which bind tightly within the pore. Here we show that several different substitutions of a positively charged amino acid residue, arginine R334, in the putative outer mouth of the CFTR pore, greatly reduce the block caused by lyotropic $Au(CN)_2^-$ ions applied to the intracellular side of the channel. Fixed positive charge at this site appears to play a role in $Au(CN)_2^-$ binding, as judged by multiple substitutions of differently charged amino acid side chains and also by the pH dependence of block conferred by the R334H mutant. However, non-charge-dependent effects also appear to contribute to $Au(CN)_2^-$ binding. Mutation of R334 also disrupts the apparent electrostatic interaction between intracellular $Au(CN)_2^-$ ions and extracellular permeant anions, an interaction which normally acts to relieve channel block. All six mutations studied at R334 significantly weakened this interaction, suggesting that arginine possesses a unique ability to coordinate ion–ion interactions at this site in the pore. Our results suggest that lyotropic anions bind tightly to a site in the outer mouth of the CFTR pore that involves interaction with a fixed positive charge. Binding to this site is also involved in coordination of multiple permeant anions within the pore, suggesting that anion binding in the outer mouth of the pore is an important aspect in the normal anion permeation mechanism.

Ion channels are defined primarily by their ionic selectivity, the ability to allow certain ions to pass at a rate which approaches that of free diffusion while at the same time effectively excluding others. In highly selective voltage-gated cation channels, selectivity is determined by selective binding of the ion of physiological interest within a localized region of the pore known as the 'selectivity filter' (Doyle *et al.* 1998; McCleskey, 1999; Zhou *et al.* 2001). The apparent incongruity between tight binding and high conductance of the permeant ion is then resolved by electrostatic repulsion between ions bound simultaneously within the pore (Doyle *et al.* 1998; McCleskey, 1999; Miller, 2000; Morais-Cabral *et al.* 2001), such that a queue of permeant ions moves through the pore in a concerted fashion (Morais-Cabral *et al.* 2001; Bernèche & Roux, 2001).

While so much has recently been revealed about the mechanism of ion permeation in highly selective K^+ channels, it is unknown how much of this mechanism may apply to anion channels. These channels are much less selective (reviewed in Dawson *et al.* 1999; Smith *et al.* 1999; Linsdell *et al.* 2000; Fahlke, 2001; Jentsch *et al.* 2002), having presumably not experienced the same kinds of evolutionary pressure to establish strong selectivity.

Nevertheless, recent evidence suggests that Cl^- channels may contain localized selectivity filters (Linsdell *et al.* 2000; Dutzler *et al.* 2002; Gong *et al.* 2002*a*) and that permeant anions bind to discrete sites within Cl^- channel pores (Dawson *et al.* 1999; Linsdell, 2001; Fahlke 2001; Dutzler *et al.* 2002; Gong *et al.* 2002*a*). Furthermore, we have shown that different permeant anions move within the pore of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in a coupled manner (Gong & Linsdell, 2003), hinting at a possible role for ion–ion interactions in the normal Cl^- permeation process.

We have used the $Au(CN)_2^-$ ion, which shows both high permeability (Smith *et al.* 1999; Gong *et al.* 2002*a*) and tight binding within the CFTR channel pore (Smith *et al.* 1999; Gong *et al.* 2002*a*; Linsdell & Gong, 2002), as a probe of intrapore anion binding sites. Here we show that a positively charged arginine residue within the sixth transmembrane region (TM6) of CFTR makes a major contribution to anion binding within the pore, provide evidence that this residue contributes to a site important for interactions between permeant anions, and examine the molecular properties of this site which underlie its roles in anion conduction.

METHODS

Experiments were carried out on baby hamster kidney (BHK) cells transiently transfected with wild-type or mutated forms of human CFTR. Mutagenesis of CFTR was carried out as described previously (Gong *et al.* 2002*a*) and verified by DNA sequencing. Macroscopic CFTR currents were recorded from excised, inside-out membrane patches, as described in the accompanying paper (Gong & Linsdell, 2003). To isolate the pore-blocking effects of intracellular $Au(CN)_2^-$, channels were 'locked' in the open state by addition of 2 mM sodium pyrophosphate (PP_i) to the intracellular solution following attainment of full PKA-stimulated current amplitude (Linsdell & Gong, 2002; Gong *et al.* 2002*a*; Gong & Linsdell, 2003). For high Cl^- concentration experiments, both pipette (extracellular) and bath (intracellular) solutions contained (mM): 150 NaCl, 2 $MgCl_2$, 10 *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonate (TES), pH 7.4. In some cases (Fig. 7), different extracellular pHs were studied; here, the pH buffer used in the extracellular solution was changed from TES to either 2-(*N*-morpholino)ethanesulfonic acid (MES) for solutions at pH 5.5, or [(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]-1-propane-sulfonic acid (TAPS) at pH 9.0. As intracellular pH was not varied, all intracellular solutions contained TES. To examine the role of extracellular anions, NaCl in the pipette solution was completely replaced by sodium gluconate or NaSCN. Solutions were adjusted to the desired pH using NaOH. Given voltages have been corrected for liquid junction potentials existing between dissimilar pipette and bath solutions as described previously (Gong & Linsdell, 2003). Other methodological details, including the modified form of the Woodhull (1973) model used to fit the blocking effects of permeant $Au(CN)_2^-$ ions, are as described in the accompanying paper (Gong & Linsdell, 2003).

In some cases (Figs 3*D*, 5, 6 and 8), the $K_d(0)$ under different ionic conditions has been compared as the ratio between individual K_d measured with one anion ($K_d(0)(X)$) and the mean K_d measured with Cl^- ($K_d(0)(Cl)$). Rectification of the macroscopic *I–V* relationship was quantified (Fig. 9*A*) by measuring the slope conductance at a voltage 50 mV more negative than the current reversal potential to that 50 mV more positive than the reversal potential, as described previously (Linsdell, 2001). The ratio of these two slope conductances was defined as the 'rectification ratio' (Fig. 9*A*); a rectification ratio greater than unity therefore indicates inward rectification of the *I–V* relationship. In some cases (Fig. 9*B*), the estimated current reversal potential has been used to estimate relative anion permeability as described in detail previously (Gong *et al.* 2002*a*). For graphical presentation of mean values, error bars represent $\pm$ S.E.M.

RESULTS

Effect of CFTR pore mutations on anion interactions

Block of the wild-type CFTR Cl^- channel pore by intracellular $Au(CN)_2^-$ ions is strongly dependent on the extracellular Cl^- concentration (Gong & Linsdell, 2003; Fig. 1), apparently due to repulsion between $Au(CN)_2^-$ ions bound within the pore and Cl^- ions bound to a site within the external entrance to the pore (Gong & Linsdell, 2003). Under
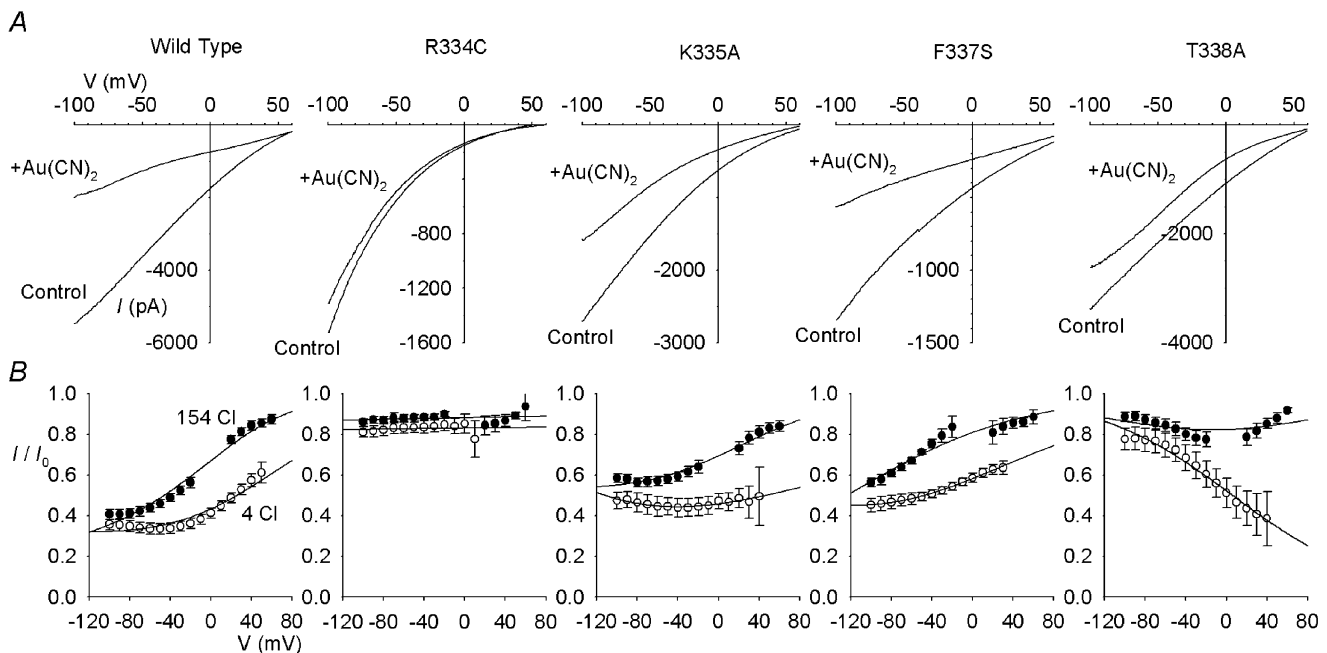

Figure 1. Extracellular Cl^--dependent $Au(CN)_2^-$ block in wild-type and mutant forms of CFTR

A, example leak subtracted CFTR macroscopic *I–V* relationships recorded with 4 mM Cl^- and 150 mM gluconate in the extracellular solution. Currents were recorded following maximal CFTR channel activation with PKA and PP_i, before (control) and after (+$Au(CN)_2$) addition of 100 μM $KAu(CN)_2$ to the intracellular solution. In each case, the scale is current in pA on the ordinate and membrane potential in mV on the abscissa. *B*, mean fraction of control current remaining (I/I_0) at different voltages following addition of 100 μM $Au(CN)_2^-$, with 154 mM Cl^- (●) or 4 mM Cl^- plus 150 mM gluconate (○) in the extracellular solution. Mean of data from 4–5 patches in each case. These mean data have been fitted by a modified version of the Woodhull (1973) model as described in the accompanying paper (Gong & Linsdell, 2003).

conditions of high extracellular Cl^- concentration, $Au(CN)_2^-$ block is weakened in the CFTR pore mutants K335A, F337S and T338A (Gong *et al.* 2002*a*), suggesting that these pore residues may contribute to lyotropic anion binding site(s) within the pore. To investigate if weakened $Au(CN)_2^-$ block in these mutants was associated with changes in the interaction between $Au(CN)_2^-$ and Cl^- ions within the pore, block by 100 μM intracellular $Au(CN)_2^-$ ions was examined under conditions of high (154 mM) and low (4 mM; Cl^- replaced by the impermeant gluconate) extracellular Cl^- concentration (Fig. 1). In spite of the altered affinity and voltage dependence of block seen in these three mutants, in each case block was still significantly weakened by extracellular Cl^- (Figs 1 and 2). In contrast, an additional mutation near the external end of TM6, R334C, not only caused a more dramatic weakening of $Au(CN)_2^-$ block than previously studied mutants (Fig. 1), but also abolished or even reversed the Cl^- dependence of both apparent affinity (as judged by the $K_d(0)$; Fig. 2*A*) and voltage dependence (as judged by the fractional electrical distance, δ; Fig. 2*B*).

Because the block of R334C-CFTR by $Au(CN)_2^-$ was so weak compared to wild-type, we further characterized this mutant using 1 mM intracellular $Au(CN)_2^-$ (Fig. 3*A* and *B*). Under these conditions, $Au(CN)_2^-$ caused a weakly voltage-dependent block which, unlike the block of wild-type CFTR, was broadly similar with different extracellular anions (Cl^-, gluconate, or SCN^-; Fig. 3*A* and *B*). Whereas in wild-type, $K_d(0)$ increases dramatically as the extracellular anion is made more lyotropic (gluconate $\rightarrow Cl^- \rightarrow SCN^-$), this trend is apparently abolished in R334C (Fig. 3*C*). The effect of changing the extracellular anion on the apparent affinity of $Au(CN)_2^-$ block in wild-type and R334C, quantified as the relative $K_d(0)$ under different ionic conditions, is directly compared in Fig. 3*D*. The dramatic effects of changing the extracellular anion observed in wild-type were practically abolished in R334C.

Characterization of R334 as an anion binding site

The effects of the mutation R334C outlined above suggest that the positively charged arginine at this position normally contributes both to a lyotropic binding site and to a site which is crucial for electrostatic interactions between extracellular permeant ions and intracellular blocking ions. To investigate the structural determinants of this site, a number of different substitutions for arginine 334 were carried out.

Previously we reported that the mutant R334A could not be expressed in BHK cells (Gong *et al.* 2002*a*); in the course of the present study, we confirmed this previous finding, but did find that, in addition to R334C, five other mutants could be studied (Fig. 4). Each of these mutants was apparently effectively locked open by PP_i (Fig. 4*A*). Block of wild-type, R334C-, R334E-, R334H-, R334K-, R334L- and R334Q-CFTR by 100 μM and 1 mM intracellular $Au(CN)_2^-$ are compared in Fig. 4*B*. Block was affected in all

mutants, depending on the ionic conditions used, but was particularly weakened in R334C, R334E and R334K (Fig. 5*A–C*). The voltage dependence of block was also reduced in all mutants studied (Fig. 5*A–C*).

Figure 5 also compares the effects of changing the extracellular anion on the apparent affinity of block in different mutants. In wild-type, block was weakened on replacing the impermeant gluconate with the permeant Cl^-, and further dramatically weakened on replacing Cl^- with the more lyotropic SCN^-. In every mutant studied, both of these
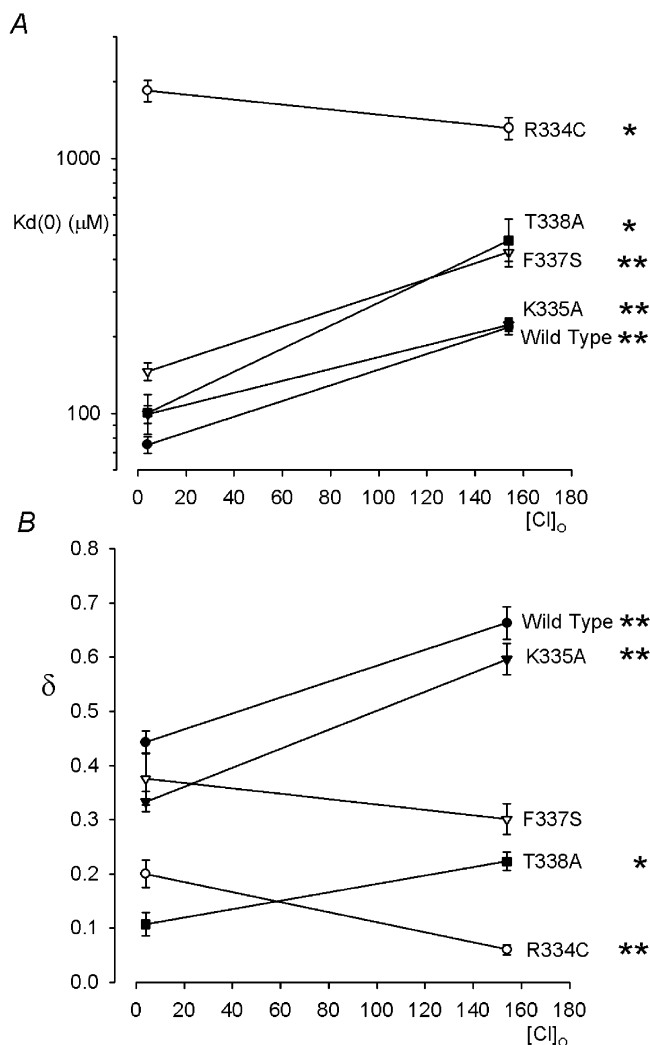

Figure 2. Chloride-dependent $Au(CN)_2^-$ block is differentially affected in different CFTR pore mutants

Mean $K_d(0)$ (*A*) and δ (*B*) were estimated by analysis of data from individual patches such as those shown in Fig. 1, as described in the accompanying paper (Gong & Linsdell, 2003). In wild-type, K335A, F337S and T338A, high extracellular Cl^- significantly weakens $Au(CN)_2^-$ block and (except in F337S) increases the fraction of the transmembrane electric field apparently experienced by the blocker. In contrast, in R334C extracellular Cl^- significantly weakens block and reduces its voltage dependence. Asterisks indicate significant difference from the data at 4 mM Cl^- (* $P < 0.05$; ** $P < 0.001$). Mean of data from 4–5 patches in each case.
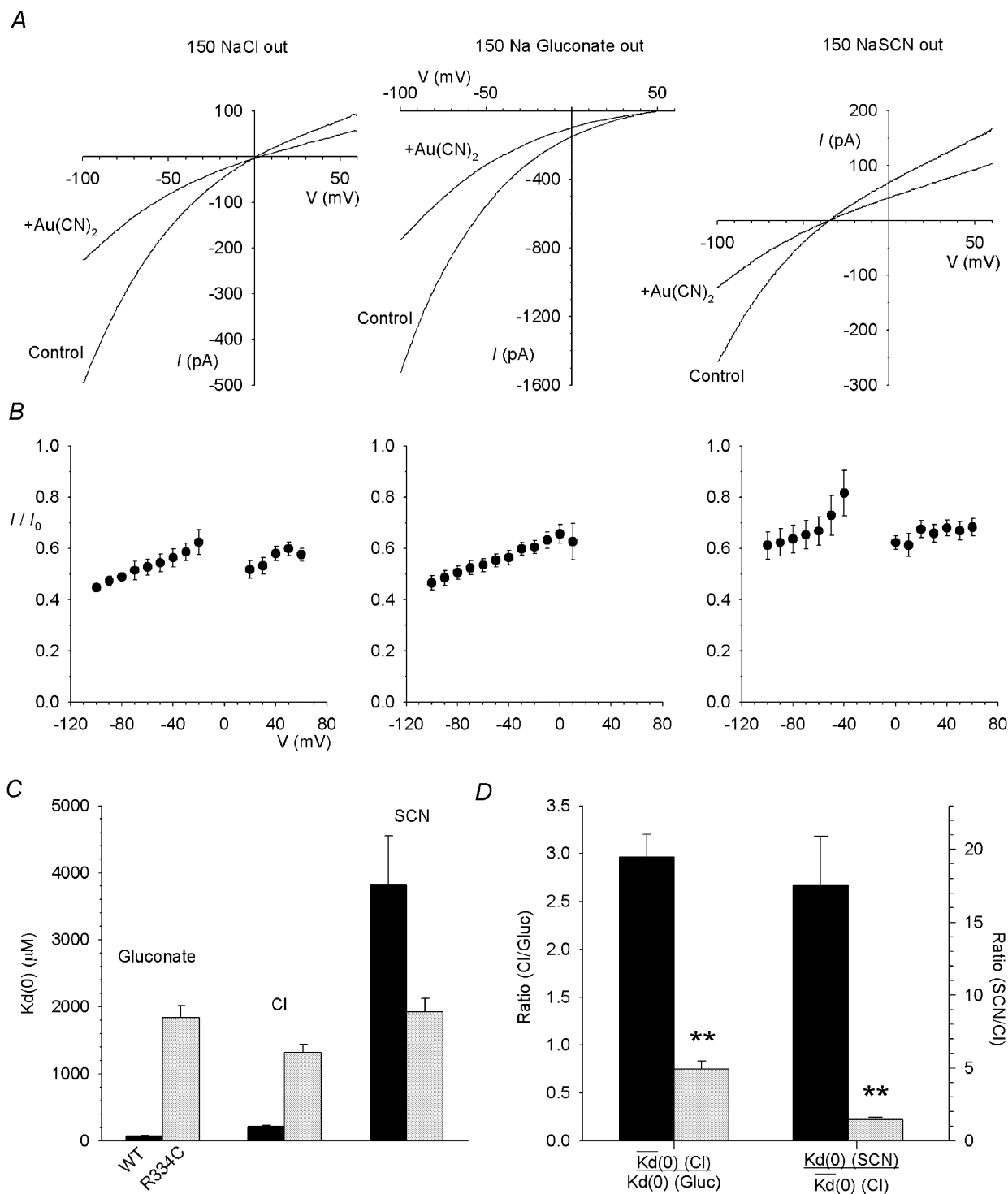
Figure 3. Au(CN)$_2^-$ block of R334C is relatively independent of the extracellular anion

A, example R334C-CFTR *I–V* relationships recorded before (control) and after (+Au(CN)$_2$) addition of 1 mM Au(CN)$_2^-$ to the intracellular solution, with 150 mM NaCl, sodium gluconate or NaSCN present in the extracellular solution. *B*, mean fraction of control current remaining (I/I_0) following addition of 1 mM Au(CN)$_2^-$ for the same ionic conditions as shown in *A*. *C*, $K_d(0)$ is strongly affected by the extracellular anion in wild-type (black bars) but not in R334C (grey bars). *D*, the effect of changing the extracellular anion (from Cl$^-$ to gluconate or from Cl$^-$ to SCN$^-$) on the strength of Au(CN)$_2^-$ block, as quantified by the ratio of $K_d(0)$ values estimated under different ionic conditions as described in Methods, is significantly reduced in R334C (grey bars) relative to wild-type (black bars) (** $P < 0.001$). Mean of data from 5 patches in each case.

effects of changing the extracellular permeant ion were greatly reduced, leading to significant decreases in the $K_d(0)$ ratio in both cases (Fig. 5D and E).

Previously we suggested that part of the voltage dependence of $Au(CN)_2^-$ block resulted from repulsive interactions with extracellular anions (Gong & Linsdell,
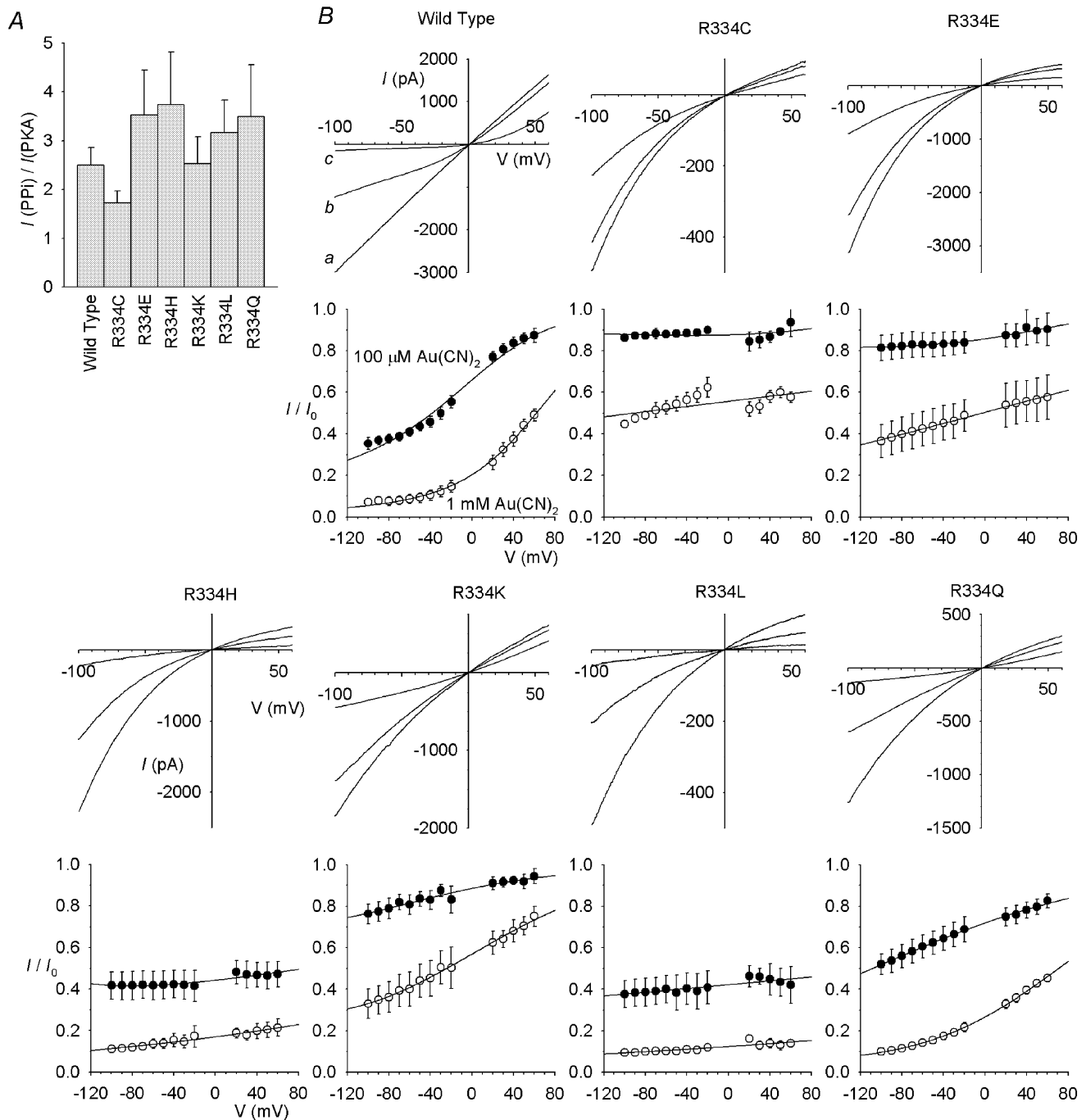

Figure 4. $Au(CN)_2^-$ block of different R334 mutants

A, mean fractional increase in macroscopic current amplitude at -100 mV following addition of 2 mM PP$_i$ for wild-type and R334 mutant CFTR channels. Mean of data from 4–5 patches. B, block by $Au(CN)_2^-$. For each CFTR variant, example I–V relationships show the blocking effects of 100 μM and 1 mM $Au(CN)_2^-$ with symmetrical Cl$^-$-containing solutions. In each case, the scale is current in pA on the ordinate and membrane potential in mV on the abscissa. In each case the current amplitude was decreased in a concentration-dependent fashion by addition of $Au(CN)_2$; for example, in wild-type, a represents control, b following addition of 100 μM $Au(CN)_2$, and c following addition of 1 mM $Au(CN)_2$. Beneath each I–V relationship, the corresponding mean I/I_0 following addition of 100 μM (○) or 1 mM (●) $Au(CN)_2^-$ is shown; this represents the mean of data from 4–5 patches, fitted as in Fig. 1B.

2003). The apparent effect of extracellular Cl^- on the affinity of $Au(CN)_2^-$ block in different mutants (as judged by the $K_d(0)$ ratio for Cl^- *versus* gluconate) was correlated with the voltage dependence of block observed with Cl^- present in the extracellular solution (δ) (Fig. 6; $r^2 = 0.832$), consistent with the idea that the same repulsive $Cl^- \text{--} Au(CN)_2^-$ interactions that affect blocker affinity similarly affect blocker voltage dependence.

A direct investigation of the role of positive charge

A previous mutagenic investigation of arginine 334 emphasized the role played by the fixed positive charge at this position, and elegantly demonstrated this effect by titrating the side chain charge in R334H by changing the external pH (Smith *et al.* 2001). Although we have studied mutants with neutral, positively charged and negatively charged side chains, the pH dependence of R334H provides a unique opportunity to examine the effect of side chain charge independently of side chain shape. We therefore examined the effect of changing extracellular pH on $Au(CN)_2^-$ block of wild-type and R334H-CFTR.

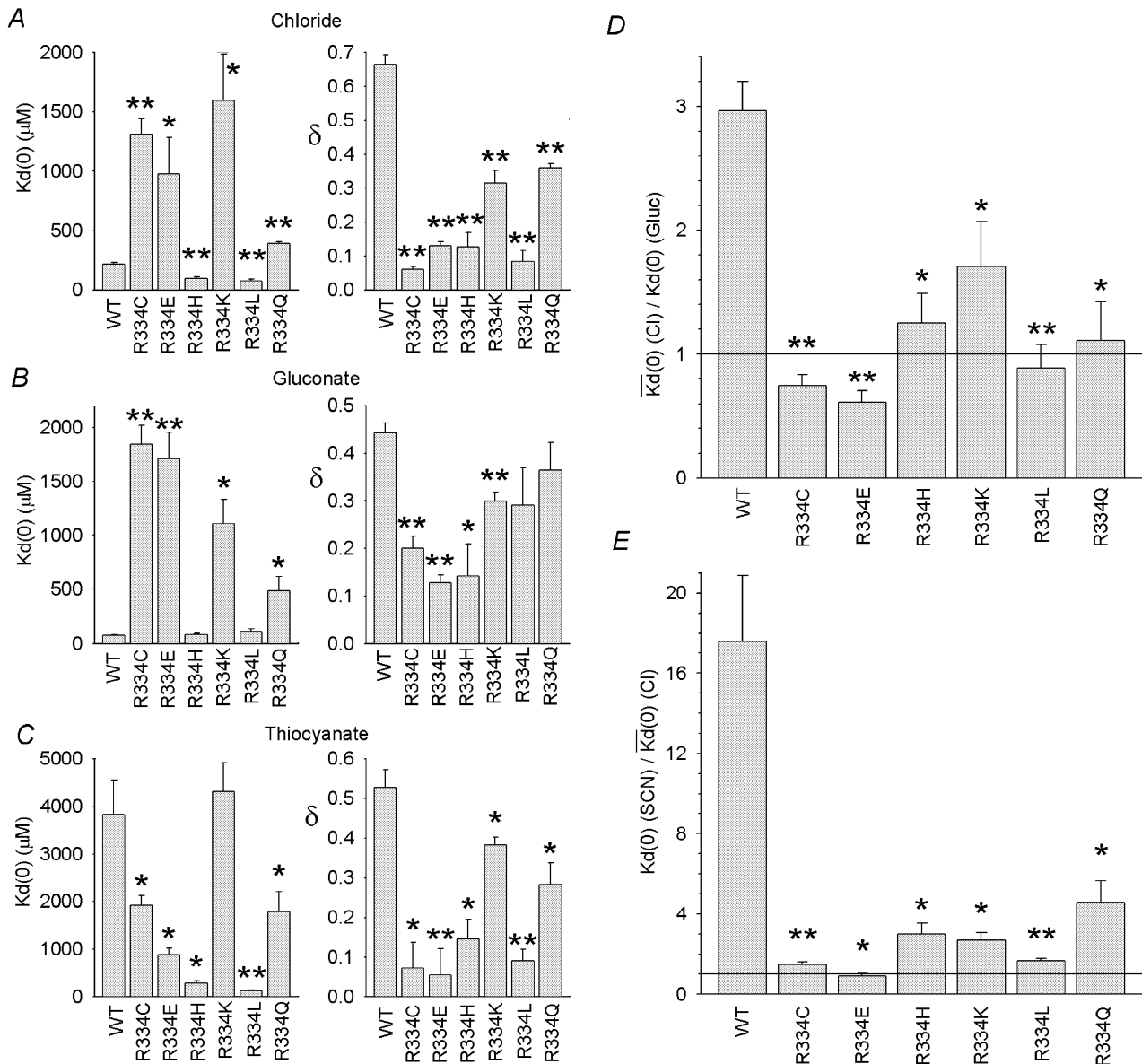As shown in Fig. 7, $Au(CN)_2^-$ blocked R334H more strongly at pH 5.5 than at pH 9.0, whereas block of wild-type was

Figure 5. Mutagenesis of R334 alters the affinity, voltage dependence and extracellular anion dependence of $Au(CN)_2^-$ block

In *A–C*, $K_d(0)$ and δ were calculated from data from individual patches with 150 mM NaCl (*A*), sodium gluconate (*B*) or NaSCN (*C*) in the extracellular solution. *D* and *E*, the effect of changing the extracellular anion (from Cl^- to gluconate (*D*) or from Cl^- to SCN^- (*E*)), quantified by the $K_d(0)$ ratio as in Fig. 3*D*, is significantly reduced in all R334 mutants relative to wild-type (* $P < 0.05$; ** $P < 0.001$). Mean of data from 3–5 patches in each case.

apparently pH independent. This suggests that the protonated histidine side chain at this position binds $Au(CN)_2^-$ more strongly than the uncharged form. However, the effect of changing the extracellular anion on the apparent affinity of $Au(CN)_2^-$ block was not strongly pH dependent in R334H (Fig. 8), and at all pHs studied the effect of changing from extracellular gluconate to Cl⁻, or from Cl⁻ to SCN⁻, was small (as judged by the $K_d(0)$ ratio) compared to the effect seen in wild-type at pH 7.4. These experiments on R334H at different pHs strongly suggest that $Au(CN)_2^-$ blocking affinity and the interaction between intracellular $Au(CN)_2^-$ ions and extracellular anions show different dependencies on side chain charge at position 334.

Other properties of R334 mutants

Our results suggest that R334 makes a strong contribution to anion binding in the CFTR pore, consistent with recent evidence that the positive charge at this position plays an important role in attracting anions to the extracellular mouth of the pore (Smith *et al.* 2001). Our experiments also revealed other properties of R334 mutants. As previously reported (Smith *et al.* 2001), all mutations at this position induced inward rectification of the *I–V* relationship (Fig. 4*B*); this effect is quantified in Fig. 9*A*. As noted by Smith *et al.* (2001), this effect was clearly charge dependent, being strongest in R334E and weak (but still

significant) in R334K. Consistent with this notion, and again as noted by Smith *et al.* (2001), rectification was pH dependent in R334H but not in wild-type (Fig. 9*A*).
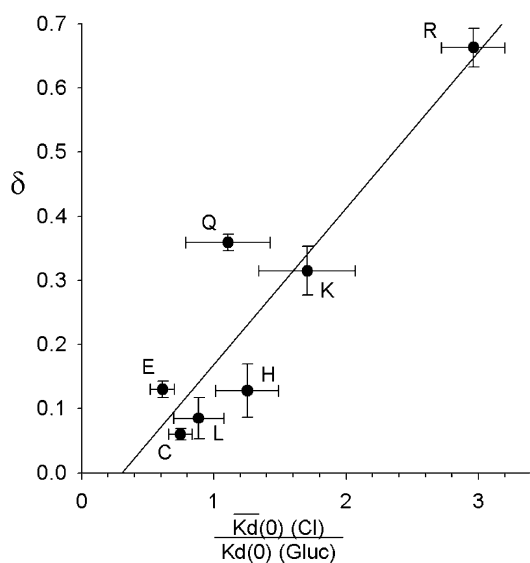

Figure 7. Extracellular pH modifies $Au(CN)_2^-$ block of R334H- but not wild-type CFTR

A, example *I–V* relationships for wild-type and R334H at extracellular pHs of 5.5 and 9.0, before (control) and after ($+Au(CN)_2$) addition of 100 μM $Au(CN)_2^-$ to the intracellular solution. In each case, the scale is current in pA on the ordinate and membrane potential in mV on the abscissa. *B*, mean I/I_0 under these same conditions, at extracellular pH 5.5 (●), 7.4 (○) and 9.0 (▼). Mean of data from 3–5 patches, fitted as in Fig. 1*B*. *C*, effect of extracellular pH on $K_d(0)$ in wild-type (●) and R334H (○).

Figure 6. Repulsive interactions with external Cl⁻ ions contribute to the voltage dependence of $Au(CN)_2^-$ block

The observed fractional electrical distance (δ) for $Au(CN)_2^-$ block in wild-type and each R334 mutant studied under high extracellular [Cl⁻] conditions appears well correlated with the effect of extracellular Cl⁻ (relative to the impermeant gluconate) on the affinity of block. The fitted line is a linear regression with a correlation coefficient (r^2) of 0.832. Parameters as described in Fig 5*A* and *D*. Different CFTR variants are identified on the plot by the amino acid present at position 334.
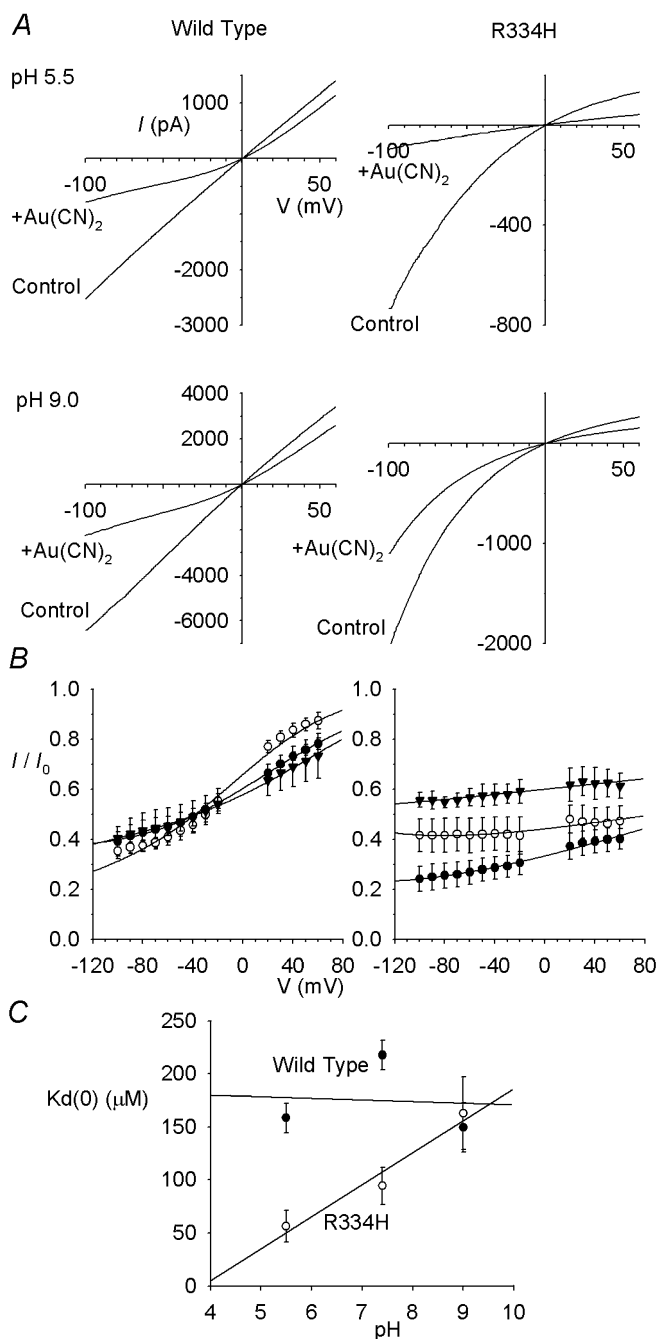
Our experiments on the effects of extracellular SCN⁻ also allowed us to estimate the SCN⁻ permeability of CFTR mutants (Fig. 9*B*). In contrast to mutations in the more central region of TM6, at F337 (Linsdell *et al.* 2000), T338 (Linsdell *et al.* 1998) and S341 (McCarty & Zhang, 2001), most substitutions for R334 had no significant effect on relative SCN⁻ permeability. However, SCN⁻ permeability was significantly increased in one mutant, R334L (Fig. 9*B*).

DISCUSSION

Arginine 334 and anion binding in the CFTR pore

The TM6 region plays a central role in forming the CFTR pore and determining its functional properties (Dawson *et al.* 1999; McCarty, 2000; Gupta *et al.* 2001; Gong *et al.* 2002*a*). Mutations within TM6 alter CFTR anion selectivity (Linsdell *et al.* 1998, 2000; McCarty & Zhang, 2001; Gong *et al.* 2002*a*) as well as the binding of permeant (Linsdell, 2001; Gong *et al.* 2002*a*) and blocking anions (McDonough *et al.* 1994; Gong *et al.* 2002*b*; Gupta & Linsdell, 2002).
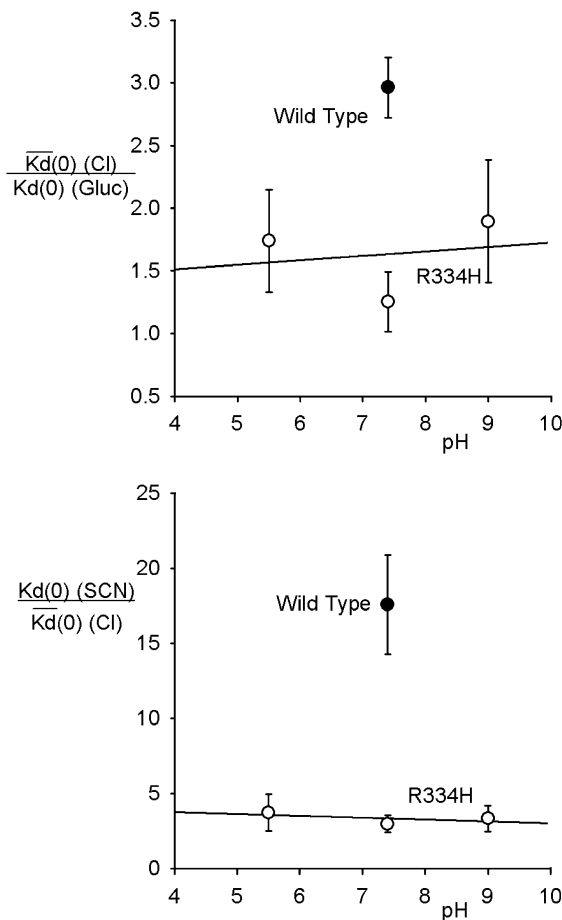

Figure 8. Extracellular pH does not affect extracellular anion dependence of $Au(CN)_2^-$ block in R334H-CFTR

The effect of changing the extracellular anion (from Cl^- to gluconate or from Cl^- to SCN^-), quantified by the $K_d(0)$ ratio as in Fig. 3*D*, appears independent of pH in R334 and is significantly reduced relative to wild-type at pH 7.4. Mean of data from 3–5 patches in each case.

Weakened binding of the highly lyotropic $Au(CN)_2^-$ ion was previously associated with mutagenesis of TM6 residues K335, F337, T338 and (to a lesser extent) I334 (Gong *et al.* 2002*a*). However, as shown in Figs 1 and 2, block by $Au(CN)_2^-$ was more dramatically altered following mutagenesis of R334, implicating this residue as contributing to a high-affinity lyotropic anion binding site within the pore. Each of the mutations R334C, R334E and
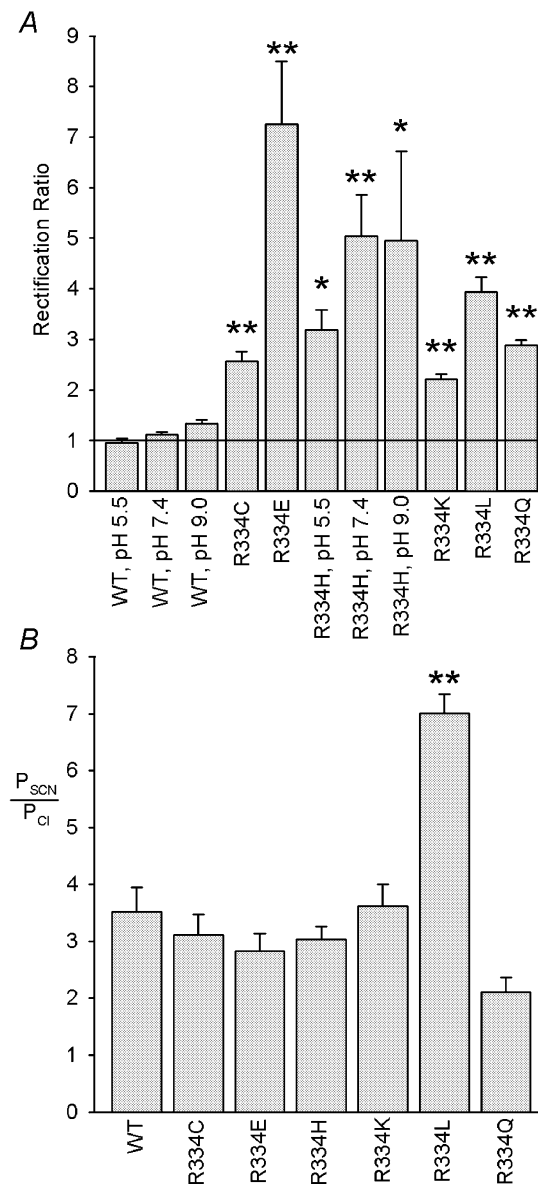

Figure 9. Mutation of R334 affects current rectification and anion selectivity

A, rectification of the macroscopic *I–V* relationship, quantified as the rectification ratio as described in Methods, is significantly increased following mutagenesis of R334 (* $P < 0.05$; ** $P < 0.001$). Furthermore, this rectification acquires pH dependence in R334H not seen in wild-type. Mean of data from 3–5 patches. *B*, relative SCN⁻ permeability, estimated from the reversal potential with 150 mM NaSCN in the extracellular solution, was unaltered in most mutants but significantly increased (** $P < 0.001$) in R334L. Mean of data from 3–5 patches.

R334K greatly decreased $Au(CN)_2^-$ affinity (Figs 4*B* and 5), increasing $K_d(0)$ 4–7-fold under conditions of high extra-cellular Cl^- (Fig. 5*A*), and 14–24-fold with the impermant gluconate ion in the extracellular solution (Fig. 5*B*), conditions under which $Au(CN)_2^-$ binding is studied in relative isolation from interactions with other anions. Thus perhaps somewhat surprisingly, combining these with our previous results (Gong *et al.* 2002*a*) suggests that the very lyotropic, highly permeant $Au(CN)_2^-$ ion binds most strongly at the putative outer mouth of the pore.

Arginine 334 may also contribute to a binding site for larger CFTR open channel blockers. Previously we showed that the R334C mutation significantly weakened block by intracellular lonidamine (Gong *et al.* 2002*b*), consistent with the idea that permeant and blocking ions may share common binding sites within the pore. While the highly permeant $Au(CN)_2^-$ is expected to have access to binding sites throughout the pore, it is not clear how a binding site near the extracellular mouth of the channel interacts with large intracellular blockers (as discussed in Gong *et al.* 2002*b*).

Assuming that $Au(CN)_2^-$ interacts strongly with Cl^- binding sites in CFTR (Linsdell & Gong, 2002), we speculate that R334 may contribute to a relatively high-affinity Cl^- binding site within the CFTR pore. No positively charged amino acid side chains were identified in the primary Cl^- binding site at the putative selectivity filter of the ClC crystal structure (Dutzler *et al.* 2002), although negatively charged side chains do bind Ca^{2+} ions in Ca^{2+} channel selectivity filters (Yang *et al.* 1993; Ellinor *et al.* 1995). Our results are consistent with R334 forming a high-affinity anion binding site in the external pore vestibule rather than the selectivity filter (see below); indeed, we find no evidence that R334 plays a major role in CFTR anion permeation selectivity (Fig. 9*B*; see below).

In addition to contributing to a high-affinity lyotropic anion binding site, R334 plays a crucial role in the inter-action between intracellular $Au(CN)_2^-$ and extracellular anions (Figs 3 and 5). In wild-type CFTR, changing the nature of the extracellular anion dramatically alters both the affinity and voltage dependence of block by intra-cellular $Au(CN)_2^-$ (Fig. 5; Gong & Linsdell, 2003). We suggested that this reflects repulsion between bound $Au(CN)_2^-$ ions and other anions bound to an extra-cellularly accessible lyotropic binding site (Gong & Linsdell, 2003). The effect of different extracellular anions on the affinity of $Au(CN)_2^-$ block is greatly weakened or lost in all R334 mutants studied (Fig. 5*D* and *E*), implicating this residue as playing a crucial role in interactions between anions bound within the pore. Furthermore, correlated effects of R334 mutations on Cl^-–$Au(CN)_2^-$ interactions and on the voltage dependence of $Au(CN)_2^-$ block (Fig. 6), support the view that such repulsive inter-actions normally contribute to blocker voltage dependence

(Gong & Linsdell, 2003). In contrast, mutation of other nearby TM6 residues associated with weakened $Au(CN)_2^-$ binding (K335A, F337S, T338A) showed similar sensitivity to extracellular Cl^- concentration to that seen in wild-type (Figs 1 and 2). We propose that anion binding to a site involving R334 is crucial in coordinating anion–anion interactions within the pore. The apparent accessibility of this site to extracellular anions (Gong & Linsdell, 2003) is consistent with models which place R334 in the outer vestibule of the CFTR pore (Cheung & Akabas, 1996; Smith *et al.* 2001; Gong *et al.* 2002*a*).

The important role played by R334 in anion binding is consistent with previous findings that mutations at this site alter single channel conductance and the shape of the macroscopic *I–V* relationship (Smith *et al.* 2001). Indeed, we find a similar effect of mutations on *I–V* rectification in inside-out membrane patches (Fig. 9*A*) to those reported by Smith *et al.* (2001) in intact *Xenopus* oocytes. Our results in inside-out patches under symmetrical ionic conditions argue that this rectification is an intrinsic property of the open channel pore and does not reflect altered block by cytoplasmic constituents, one possibility raised by Smith *et al.* (2001).

In spite of its clear importance in anion binding, R334 does not appear to play a major role in lyotropic anion permeation selectivity (Fig. 9*B*). This is consistent with our previous suggestion that selectivity is determined pre-dominantly over a short region of the pore located more cytoplasmically than R334 (Linsdell *et al.* 2000; Gong *et al.* 2002*a*) and that some anion binding sites within the pore are not intimately involved in the selectivity process (Gong *et al.* 2002*a*).

Separating the different effects of mutagenesis at arginine 334

The effects of mutagenesis of R334 on the apparent affinity of $Au(CN)_2^-$ block and on its dependence on the extra-cellular anion are both consistent with disruption of an important anion binding site in the external mouth of the pore. Since there is broad agreement that the side chain at R334 lines the aqueous lumen of the CFTR pore (Cheung & Akabas, 1996; Smith *et al.* 2001), unlike the situation for another arginine residue in TM6, R347 (Cheung & Akabas, 1996; Cotten & Welsh, 1999; Smith *et al.* 2001), we assume that anion–amino acid side chain interactions dominate the permeation phenotype conferred by different R334 mutations, although as with all mutagenesis experiments, more indirect effects cannot be ruled out.

With either Cl^- or gluconate in the extracellular solution, $Au(CN)_2^-$ block was most dramatically weakened in the mutants R334C, R334E and R334K, which involve replace-ment of the positively charged arginine side chain with one neutral side chain (cysteine), one negatively charged side chain (glutamate) and one positively charged side chain

(lysine). This suggests that charge does not play a dominant role in determining $Au(CN)_2^-$ affinity at this site. In R334H, positive charge does appear to enhance $Au(CN)_2^-$ binding, since low pH, which is expected to favour protonation of this side chain (see also Smith *et al.* 2001), increases the apparent affinity of block (Fig. 7).

All mutations at R334 interfered with the ability of different extracellular anions to affect the apparent affinity of $Au(CN)_2^-$ block (Fig 5*D* and *E*), presumably due to a disruption of anion–anion interactions within the pore (see above). Thus, among the amino acids studied at position 334, the wild-type arginine appears unique in its ability to confer strong repulsive interactions between $Au(CN)_2^-$ in the pore and extracellular anions.

The different sensitivity of different parameters of pore function on amino acid side chain charge at position 334 suggests that these aspects of the permeation mechanism may not be strongly interdependent or even controlled by similar structural features of the pore. These different effects of side chain charge are clearly demonstrated in R334H, which shows pH-dependent rectification (Fig. 9*A*) and $Au(CN)_2^-$ affinity (Fig. 7) but pH-independent interactions between intracellular $Au(CN)_2^-$ and extracellular anions (Fig. 8). The fact that tight $Au(CN)_2^-$ binding and strong $Au(CN)_2^-$–anion interactions are separable by mutagenesis is also evident in R334H and R334L (Fig. 5).

The specific role of arginine

Of the six R334 mutants studied, none could reproduce the strong anion–anion interactions seen in wild-type (Fig. 5*D* and *E*) or the resulting voltage dependence of $Au(CN)_2^-$ block (Fig. 6). This implies a specific role for arginine in coordinating interactions between anions bound simultaneously in the pore. Arginine has frequently been proposed as being particularly well suited to form anion binding sites in proteins (Riordan, 1979; Tabcharani *et al.* 1997; Dawson *et al.* 1999). We hypothesize that the large, highly hydrated surface area (Radzicka & Wolfenden, 1988) and diffuse positive charge of the guanidinium group of arginine gives it a unique ability to coordinate the binding of multiple anions within the pore. This could occur, for example, if the guanidinium group, which contains five hydrogen bond donors in a planar array (Dawson *et al.* 1999), was able to interact simultaneously with two different anions. However, we previously found that extracellular anions much larger than Cl^-, such as ClO_4^-, were able to interact with intracellular $Au(CN)_2^-$ (Gong & Linsdell, 2003). Alternatively, the long arginine side chain may allow it to bind a single anion in close proximity to a second anion bound to a distinct but nearby

binding site; the nearby TM6 residues K335, F337 and T338 all appear to be involved in $Au(CN)_2^-$ binding in the CFTR pore (Gong *et al.* 2002*a*).

Implications for the permeation mechanism in the CFTR pore

Our results suggest that R334, a pore-lining TM6 residue predicted to lie in the outer vestibule of the CFTR pore (Cheung & Akabas, 1996; Smith *et al.* 2001; Gong *et al.* 2002*a*), contributes a relatively high-affinity lyotropic anion binding site in the pore. Furthermore, this binding site appears to play a crucial role in the ability of extracellular anions to interact with blocking anions bound within the pore. We propose that R334 does, in fact, contribute to the extracellularly accessible lyotropic anion binding site predicted by the accompanying paper (Gong & Linsdell, 2003). Although other TM6 residues located more intracellularly than R334 also appear to contribute to lyotropic anion binding (e.g. K335, F337, T338; Fig. 1; Gong *et al.* 2002*a*), the effects of mutations studied to date suggest that the highly lyotropic $Au(CN)_2^-$ anion binds most strongly at R334.

We proposed that electrostatic repulsion between anions bound simultaneously within the CFTR pore may help maximize the rate of Cl^- permeation (Gong & Linsdell, 2003), a mechanism analogous to that which occurs in K^+ channel (Doyle *et al.* 1998; Morais-Cabral *et al.* 2001) and Ca^{2+} channel pores (McCleskey, 1999), and which has been suggested on structural grounds in ClC-type Cl^- channels (Dutzler *et al.* 2002). This mechanism appears to involve R334, but is not conferred simply by tight $Au(CN)_2^-$ binding. Within K^+ channel pores, K^+ binding sites are spaced along the axis of the pore, both within and on either side of the selectivity filter, and ions move between these sites in a concerted fashion (Morais-Cabral *et al.* 2001; Zhou *et al.* 2001; Bernèche & Roux, 2001). In Ca^{2+} channel pores, a ring of negatively charged glutamate residues within the selectivity filter can coordinate one Ca^{2+} ion tightly or two Ca^{2+} ions weakly, leading to exit (permeation) of bound Ca^{2+} ions from doubly occupied pores (Yang *et al.* 1993; Ellinor *et al.* 1995; McCleskey, 1999). Calcium ions do not appear to bind tightly outside the selectivity filter region (Cibulsky & Sather, 2000). Although it is not at present clear which of these two mechanisms most closely resembles that of Cl^- permeation in the CFTR pore, it is interesting to note that R334 does not appear to be intimately involved in the selectivity of anion permeation (Fig. 9*B*) and probably confers tight anion binding on the extracellular side of the anion selectivity filter (Gong *et al.* 2002*a*).

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