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Structural and ionic determinants of 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid block of the CFTR chloride channel

*,[1]Kenneth B. Walsh, [1]Kathryn J. Long & [1]Xufeng Shen

[1]Department of Pharmacology, University of South Carolina, School of Medicine, Columbia, South Carolina, SC 29208, U.S.A.

1 The goals of this study were to identify the structural components required for arylaminobenzoate block of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel and to determine the involvement of two positively charged amino acid residues, found within the channel, in drug binding.

2 Wild-type and mutant CFTR chloride channels were expressed in *Xenopus* oocytes and CFTR currents measured using the two microelectrode voltage clamp. Block of the wild-type CFTR current by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) occurred in a voltage-dependent manner with preferential inhibition of the inward currents (K_d = 166 μM at −90 mV).

3 Removal of the phenyl ring from the aliphatic chain of NPPB, with the compound 2-butylamino-5-nitrobenzoic acid, caused only a small change in CFTR inhibition (K_d = 243 μM), while addition of an extra phenyl ring at this position (5-nitro-2-(3,3-diphenylpropylamino)-benzoic acid) increased drug potency (K_d = 58 μM). In contrast, removal of the benzoate ring (2-amino-4-phenylbutyric acid) or the 5-nitro group (2-(3-phenylpropylamino)-benzoic acid) of NPPB severely limited drug block of the wild-type channel.

4 NPPB inhibition of CFTR currents in oocytes expressing the mutants K335E and R347E also occurred in a voltage-dependent manner. However, the K_ds for NPPB block were increased to 371 and 1573 μM, for the K335E and R347E mutants, respectively.

5 NPPB block of the inward wild-type CFTR current was reduced in the presence of 10 mM of the permeant anion SCN^-.

6 These studies present the first step in the development of high affinity probes to the CFTR channel.

Keywords: CFTR Cl^- channels; arylaminobenzoates; site-directed mutagenesis; SCN^-

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; NPPB 5-nitro-2-(3-phenylpropylamino)-benzoate

Introduction

Cystic fibrosis is an autosomal-recessive disease that results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Anderson *et al*., 1992; Riordan, 1994). In epithelial cells from a variety of tissues, CFTR functions as a protein kinase A (PKA)-activated Cl^- channel (Kartner *et al*., 1991; Anderson *et al*., 1991b). CFTR exists in roughly two equal structural halves with each half of the protein consisting of six membrane spanning domains (M1–6 or M7–12) and a nucleotide binding domain (NBD1 or NBD2) (Riordan *et al*., 1989). At least two or more of the membrane spanning domains are believed to contribute in forming the ion conducting pore of the channel (Anderson *et al*., 1991a; Tabcharani *et al*., 1993; McDonough *et al*., 1994; Cheung & Akabas, 1997; Mansoura *et al*., 1998). Site directed mutagenesis experiments have demonstrated that positively charged arginine and lysine residues in the M6 domain function as major determinants of anion selectivity (Anderson *et al*., 1991a; Tabcharani *et al*., 1997).

Although the CFTR Cl^- channel has been widely studied in both human epithelial tissues and in heterologous cells expressing the CFTR gene (Anderson *et al*., 1992; Riordan, 1994), little quantitative data is available concerning the pharmacology of this channel. Development of a high affinity ligand to the channel would aid in mapping the structural architecture of the pore region of the protein and could lead to the discovery of compounds which stimulate chloride transport. Most studies of CFTR in this area have focused on one of two classes of compounds: arylaminobenzoates such as diphenylamine-2-carboxylate (DPC) (Anderson *et al*., 1992; McCarty *et al*., 1993) or sulfonylureas (ATP-sensitive K^+ channel blockers) such as tolbutamide and glibenclamide (Sheppard & Welsh, 1992; Sheppard & Robinson, 1997). DPC blocks the CFTR Cl^- when applied at relatively high (200 μM–3 mM) concentrations (Anderson *et al*., 1992). Wangemann *et al*. (1986) modified the structure of DPC, to produce a group of arylaminobenzoate compounds that varied in the phenyl to benzoate group, carbon chain length and contained a nitro group at the 5′ position of the benzoate ring (Wangemann *et al*., 1986). One of these arylaminobenzoates, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), was identified as a potent blocker of Cl^- channels in the thick ascending limb of the kidney (Wangemann *et al*., 1986; Tilmann *et al*., 1991).

Recently we reported that NPPB blocks the cardiac isoform of CFTR in a voltage- and pH-dependent manner (Walsh & Wang, 1998). In the present study we sought to identify structural components of NPPB that are involved in channel block and to determine if NPPB binds within the pore of the CFTR channel. For this purpose, the epithelial CFTR channel was expressed in *Xenopus* oocytes and the inhibitory effect of various NPPB analogues quantified. In addition, it was determined if two positively charged amino acid residues, found in the M6 domain of CFTR, are involved in NPPB binding to the channel. Finally, we studied the effect of the permeant anion SCN^- in modulating NPPB inhibition.

*Author for correspondence; E-mail: walsh@med.sc.edu

Methods

Preparation of wild-type and mutant CFTR mRNA

The cDNA (in Bluescript vector) for the wild-type human epithelial CFTR channel was generously supplied by Dr Alan Smith (Genezyme Corp.), and the K335E and R347E mutants obtained from Dr K Kunzelmann (Albert-Ludwigs University, Freiburg, Germany). These mutations were confirmed by sequencing prior to experimentation. For *in vitro* RNA transcription, the vectors were linearized with either *Sma*I (wild-type) or *Kpn*I (mutants). CFTR transcripts were prepared using the mMessage mMachine kit (Ambion Inc.).

Expression and measurement of CFTR channels in Xenopus oocytes

Ovarian lobes were removed from fully anaesthetized adult frogs (*Xenopus*I) and oocytes isolated in OR 2 solution containing (in mM): NaCl 83, KCl 2, $MgCl_2$ 1 and HEPES 5, pH 7.5, with 2 mg ml^{-1} collagenase (type A; Boehringer Mannheim). Oocytes were stored on a rotary shaker in ND-96 solution containing (in mM): NaCl 86, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 Na-pyruvate 2.5 and HEPES 5, pH 7.5, at 18°C. Twenty-four hours after isolation, stage V and VI oocytes were injected with 10–25 nl of CFTR mRNA (0.1–0.2 mg ml^{-1}) using a microinjector (Drummond Scientific Co.). CFTR Cl^- currents were measured 1 to 3 days following injection using a TEV 200 two microelectrode voltage clamp (Dagan Corp.). Electrodes (Dagan LE 16 glass) were filled with a 3 M KCl solution and had resistances of 0.5–2 MΩ. With the exception of the NaSCN substitution experiments (Figure 8), all recordings were conducted in a Ca^{2+}- and pyruvate-free ND-96 solution. Oocytes were maintained at a holding potential of −40 mV and voltage steps applied to potentials in the range of −100 to +60 mV. CFTR currents were activated by application of a 'cyclic AMP cocktail' containing forskolin (10 μM), 8-chlorphenylthio (8-CPT) cyclic AMP (200 μM) and 3-isobutyl 1-methylxanthine (IBMX) (500 μM) (all obtained from Sigma Chemical Co.) to stimulate protein kinase A.

Preparation and use of NPPB analogues

The structures of the drugs 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 2-butylamino-5-nitrobenzoic acid (BANB), 5-nitro-2-(3,3-diphenylpropylamino)-benzoic acid (NDPB), 2-(3-phenylpropylamino)-benzoic acid (PPAB), 2-amino-4-phenylbutyric acid (APB) and 5-nitro-2-(4-phenylbutylamino)-benzoate (NPBB) are shown in Figure 1. The compounds NPPB, BANB, NDPB, PPAB and NPBB were generously supplied by Dr Rainer Greger of Albert-Ludwigs University in Freiburg, Germany. APB was obtained from Aldrich Chemical Co. Stock solutions of the drugs were prepared in 100% DMSO and diluted into the external solution so that the final volume of DMSO was ⩽0.1%.

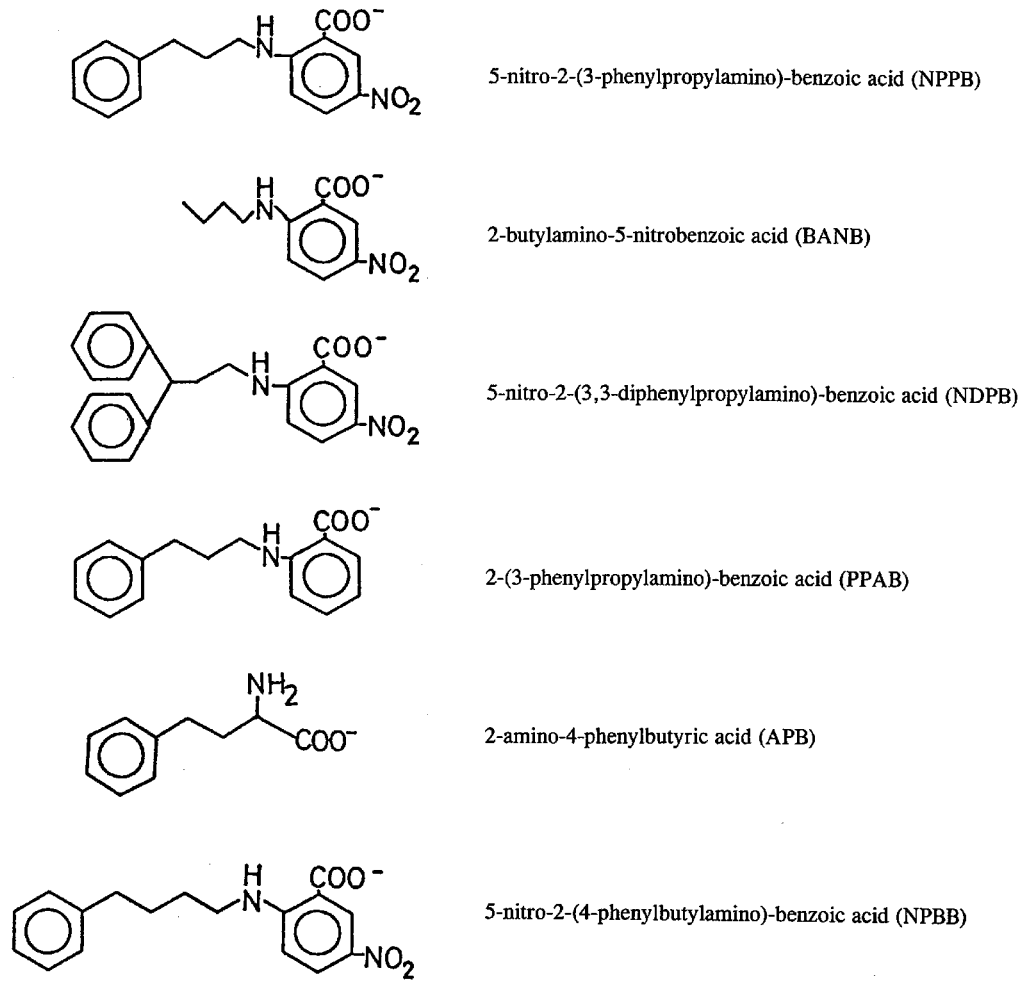

Figure 1 Structure of NPPB analogues used in this study.

Addition of 0.1% DMSO alone to the 'cyclic AMP cocktail' caused no change in the amplitude of the CFTR currents. CFTR channel block was quantified at 5 min following drug addition to the recording chamber. This represented a time when drug block had reached a steady state.

The voltage-dependence of CFTR channel block was determined by fitting the relationship between the K_d of NPPB ($I_d/I_o = K_d/(K_d + [\text{NPPB}])$ with I_o and I_d representing the CFTR current amplitudes measured before and after the addition of the drug, respectively), determined at various membrane potentials, and the voltage (V) with the equation:

$$K_d(\text{V}) = K_d(0) * \exp(z\theta\text{FV}/\text{RT}) \quad (1)$$

where $K_d(0)$ is the K_d at 0 mV, θ is the electrical distance sensed by the blocker, F is Faraday's constant, R is the gas constant, and T is the temperature. In all the calculations it was assumed that the valence (z) was −1 and that there was a single binding site for the arylaminobenzoates.

All experiments were conducted at room temperature (21–23°C). Averaged values presented are means ± s.e.mean. Where appropriate, statistical significance was estimated using Student's *t*-test for unpaired observations.

Results

NPPB block of the CFTR Cl^- current

Figure 2 shows an example of CFTR Cl^- currents measured in a *Xenopus* oocyte previously injected with epithelial CFTR mRNA. The CFTR currents were activated by addition of a 'cyclic AMP cocktail' containing 10 μM forskolin, 200 μM 8-CPT cyclic AMP and 500 μM IBMX (cyclic AMP) (Figure 2). Exposure of water-injected or non-injected oocytes to the 'cyclic AMP cocktail' failed to increase the membrane conductance (results not shown). The CFTR currents displayed an outward rectifying current versus voltage (I/V) relationship under the conditions of the experiments (Figure 3, left panel). This rectification more than likely resulted from the low intracellular [Cl^-] in the oocytes (Cunningham *et al*., 1992; McCarty *et al*., 1993). The CFTR currents ranged in size from −2 to −5 μA at −90 mV and from 3 to 10 μA at +60 mV. Similar cyclic AMP-activated Cl^- currents have previously been described in oocytes injected with CFTR mRNA (Cunningham *et al*., 1992; McCarty *et al*., 1993; Cheung & Akabas, 1996).

Addition of 100 μM 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) to the CFTR-injected oocytes resulted in a voltage-dependent block of the Cl^- currents (Figures 2 and 3). Overall in five oocytes examined, 100 μM NPPB decreased the inward (at −90 mV) and outward (at +60 mV) CFTR current by 40 ± 2% and 14 ± 3%, respectively. Concentration versus response curves for NPPB were constructed for several membrane potentials and the K_d determined as illustrated for the data obtained at −90 mV (see Figure 4). The right panel of Figure 3 plots the relationship between the K_d (V) and the membrane potential. The electrical distance sensed by the NPPB molecule, θ, and dissociation constant at 0 mV ($K_d(0)$) were determined using the Woodhull equation described in the Methods section (Woodhull, 1973). An θ of 0.24 was calculated with the orientation from the inside membrane and the $K_d(0)$ was 387 μM.

Effect of chemical modifications on NPPB potency

Previous studies have shown that slight modifications to the structure of NPPB change the potency for Cl^- current inhibition in the thick ascending limb of the kidney (Wangemann *et al*., 1986). Since NPPB inhibits the CFTR current, it was important to determine what structural components of the drug molecule are necessary for channel block. Figure 4 compares the concentration versus inhibition curve for NPPB with curves obtained for the drugs 2-butylamino-5-nitrobenzoic acid (BANB), 5-nitro-2-(3,3-diphenylpropylamino)-benzoic acid (NDPB), 2-(3-phenylpropylamino)-benzoic acid (PPAB), 2-amino-4-phenylbutyric acid (APB) and 5-nitro-2-(4-phenybutylamino)-benzoate (NPBB). Since block of CFTR current by all the drugs was voltage-dependent, the data for the concentration versus inhibition curves were recorded at −90 mV. NPPB blocked the CFTR channel with a K_d of 166 μM. Removal of the phenyl ring from NPPB in the drug BANB caused only a slight change in the potency of channel block (K_d = 243 μM) (Figure 4, left panel). In contrast, addition of an extra phenyl ring (NDPB) at this position resulted in a 3 fold

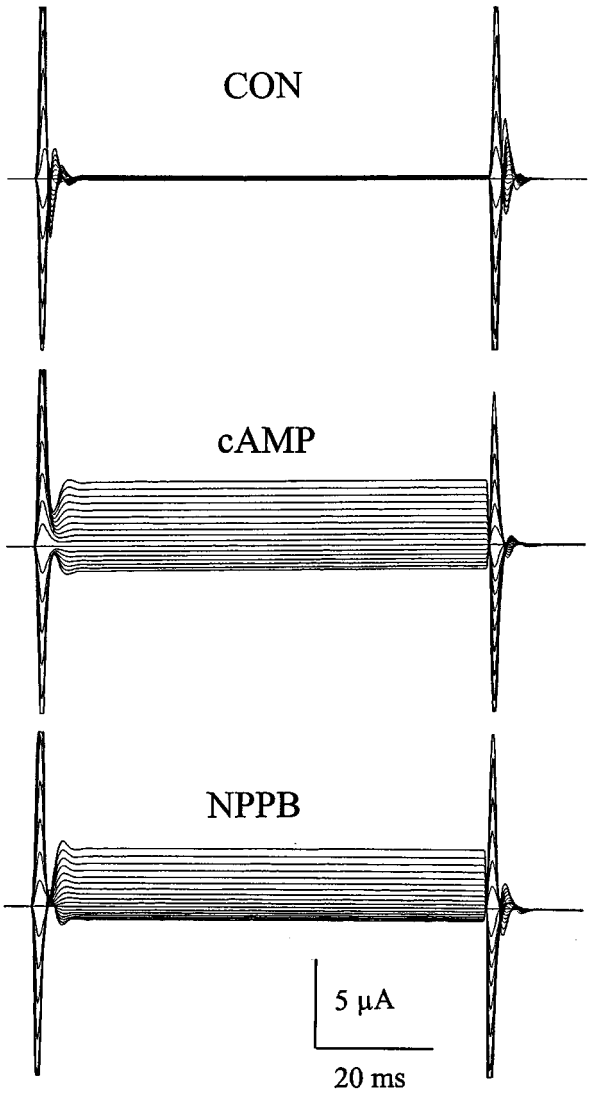

Figure 2 Inhibition of wild-type CFTR currents by NPPB. Membrane currents measured from a *Xenopus* oocyte, previously injected with CFTR mRNA, during 80 ms voltage steps applied to various membrane potentials. Currents were recorded under basal conditions (CON), in the presence of the cyclic AMP cocktail solution (forskolin-10 μM, 8-CPT cyclic AMP-200 μM and IBMX-500 μM) and 5 min after the addition 100 μM NPPB.

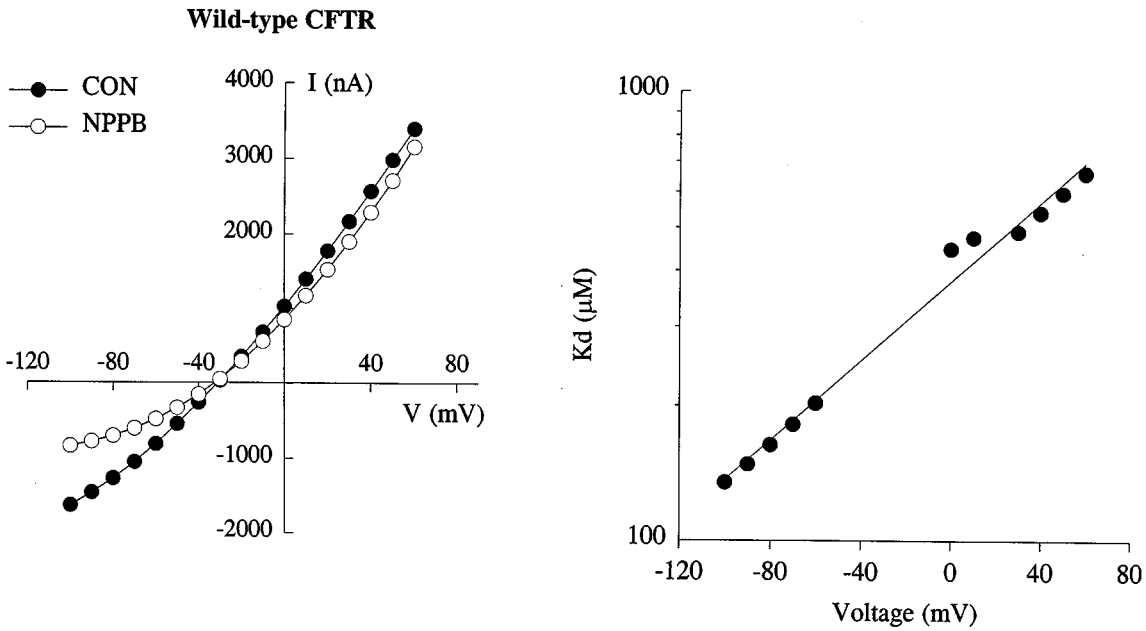

Figure 3 Voltage-dependent block of the wild-type CFTR channel by NPPB. Left panel: I/V relationship for the CFTR current measured in the presence and absence of 100 μM NPPB. In this experiment NPPB caused decreases of 47 and 7% in the current amplitude measured at -90 and $+60$ mV, respectively. Oocyte 8202. Right panel: Relationship between the K_d and the membrane potential. The K_d was determined at each potential as described in the Methods section and the relationship fit with the equation $K_d(V) = K_d(0) * \exp(z\theta FV/RT)$. The fitted line had values of 0.24 for the θ and 387 μM for the $K_d(0)$.

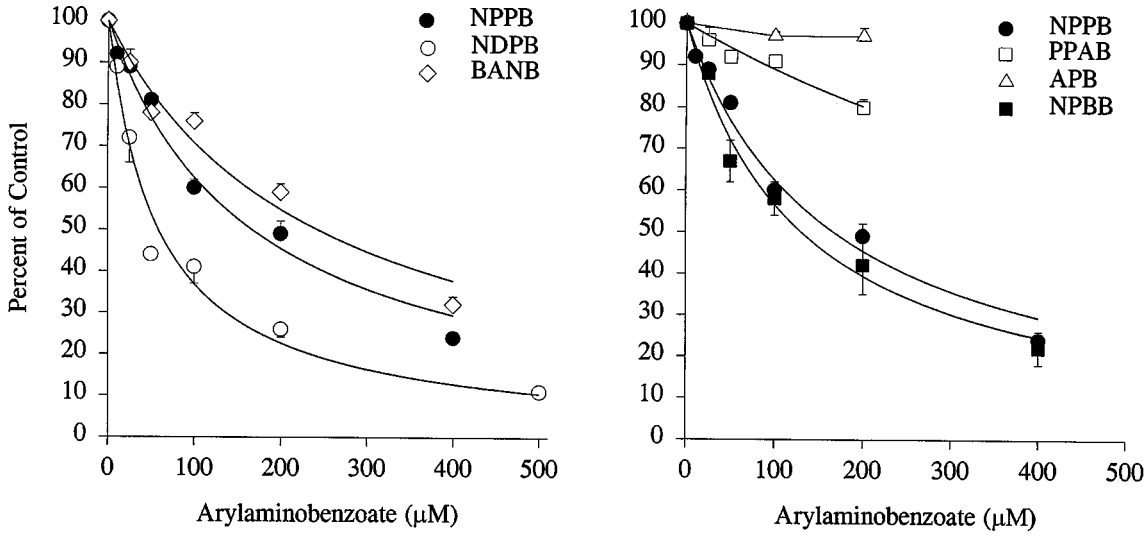

Figure 4 Concentration versus response curves for inhibition of the wild-type CFTR channel by NPPB analogues. Each point represents the mean$\pm$s.e.mean of three to six experiments. Data were recorded at -90 mV. The theoretical curves for the data are given by 1-(K_d/{K_d+[drug]}) with K_ds of 58, 131, 166, 243 and 815 μM for NDPD, NPBB, NPPB, BANB, and PPAB, respectively, providing the best least squares fit to the points. The APB data points were connected by straight lines.

stronger inhibition of the CFTR current (K_d= 58 μM) (Figure 4, left panel). Removal of or substitutions to the benzoate ring of NPPB greatly weakened drug block of the channel. This effect was particularly striking with the compound APB which lacked the benzoate ring and was without inhibitory action at 100 and 200 μM concentrations (Figure 4, right panel). In addition, removal of the 5-nitro group from NPPB, in the drug PPAB, caused a large decrease in drug potency (K_d=815 μM) (Figure 4, right panel). Lengthening the carbon chain separating the phenyl and benzoate rings with NPBB caused almost no change in drug potency (K_d=131 μM) (Figure 4, right panel).

Effect of NPPB on K335E and R347E CFTR mutants

The pKa of NPPB is close to 4.5 (Wangemann *et al.*, 1986; Walsh & Wang, 1998), and thus the drug molecules are predominately charged (>99%) in the ND-96 solution at pH 7.5. Since the negatively charged drug may interact with positively charged amino acid residues in the pore of the CFTR channel, we examined the effect of NPPB on the mutants K335E and R347E. As shown in Figures 5 and 6, the I/V relationships for currents recorded in oocytes expressing the mutant CFTR channels varied somewhat from the I/V

relationship of the wild-type channel (Figure 3). The K335E channel displayed a more linear I/V relationship (Figure 5) and the R347E channel a more outward-rectifying I/V relationship (Figure 6) than that measured with the wild-type channel (Figure 3). This is consistent with previous measurements demonstrating changes in the I/V relationships for channels containing mutations in the M6 region (Anderson *et al*., 1991a; McDonough *et al*., 1994). NPPB was less effective in blocking the CFTR currents in oocytes expressing the K335E and R347E channels. The K_d for NPPB block of the current was increased from 166 μM for the wild-type to 371 and 1573 μM for the K335E and R347E mutants, respectively (Figures 5 and

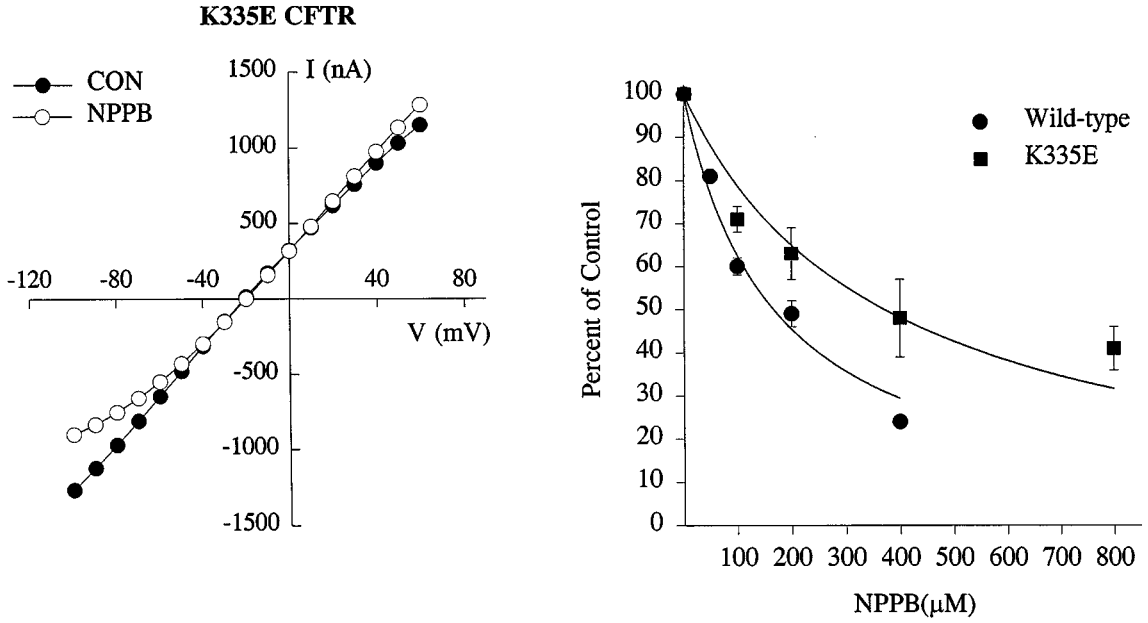

Figure 5 Effect of NPPB on the K335E CFTR channel. Left panel: I/V relationship for the CFTR current measured in the presence and absence of 100 μM NPPB. In this experiment changes of -26 and $+10\%$ were recorded in the presence of NPPB for currents measured at -90 and $+60$ mV, respectively. Oocyte O7D05. Right panel: concentration versus response curve for inhibition of the wild-type and K335E channels by NPPB. Data were recorded at -90 mV. The wild-type data are taken from Figure 4. For the K335E data, each point represents the mean$\pm$s.e.mean of three to five experiments. The theoretical curve for the K335E data provided a K_d of 371 μM.

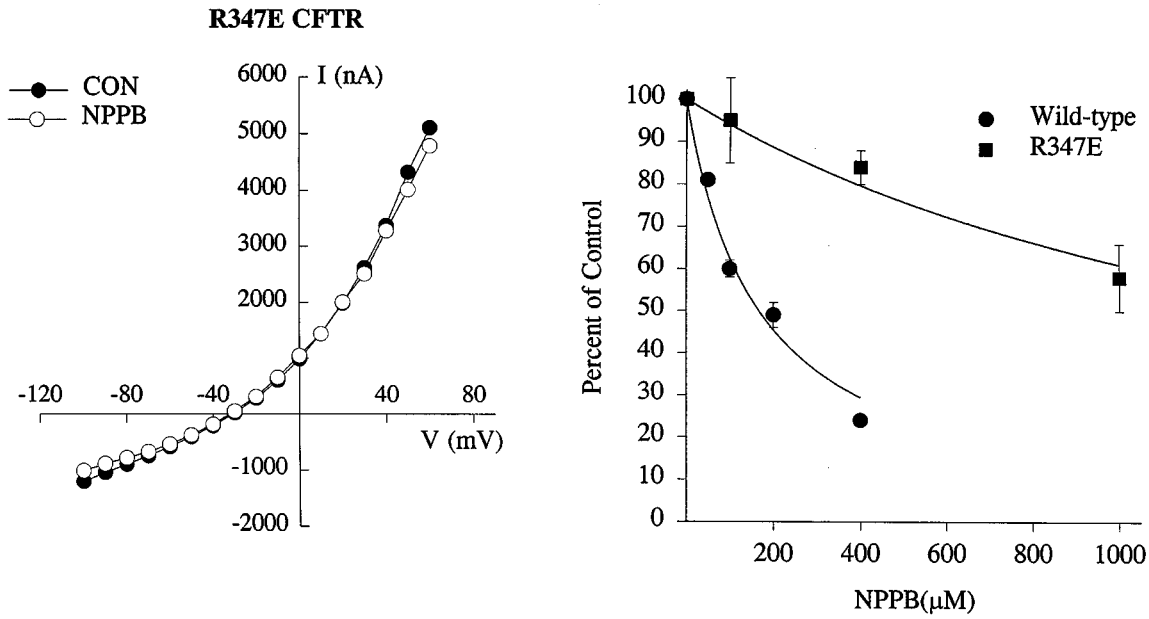

Figure 6 Effect of NPPB on the R347E CFTR channel. Left panel: I/V relationship for the CFTR current measured in the presence and absence of 100 μM NPPB. In this experiment NPPB caused decreases of 15 and 6% in the current amplitude measured at -90 and $+60$ mV, respectively. Oocyte O7N19. Right panel: concentration versus response curve for inhibition of the wild-type and R347E channels by NPPB. Data were recorded at -90 mV. The wild-type data are taken from Figure 4. For the R347E data, each point represents the mean$\pm$s.e.mean of three to five experiments. The theoretical curve for the R347E data provided a K_d of 1573 μM.

6). Although the sensitivity of the mutant channels to NPPB was reduced, both the K335E and R347E mutants displayed a voltage-dependence to NPPB block that was similar to the wild-type channel (Figure 7). The slopes of the lines obtained from the relationship between the fractional block (I_d/I_o) and voltage had values of 0.23 (wild-type), 0.24 (K335E) and 0.30 (R347E).

Effect of NPPB on CFTR currents in a SCN^- bathing solution

Partial substitution of external Cl^- with SCN^- reduces the conductance of the CFTR channel (Tabcharani *et al*., 1993; Hipper *et al*., 1995; Linsdell *et al*., 1997). In order to determine if the inhibitory action of NPPB is modulated by SCN^-, 10 mM of the NaCl in the ND-96 bathing solution was replaced with 10 mM NaSCN. When compared with the normal 86 mM NaCl-containing solution, NPPB block of the inward wild-type CFTR current was significantly reduced ($P<0.05$) in the presence of SCN^- (Figure 8, right panel). However, NPPB block of the outward current was not significantly changed ($P>0.05$) in the SCN^- solution (Figure 8, right panel). This decrease in NPPB inhibition at negative potentials with external SCN^-, gave the overall appearance to the I/V relationship of a weakening in the voltage-dependence of NPPB block (Figure 8, left panel).

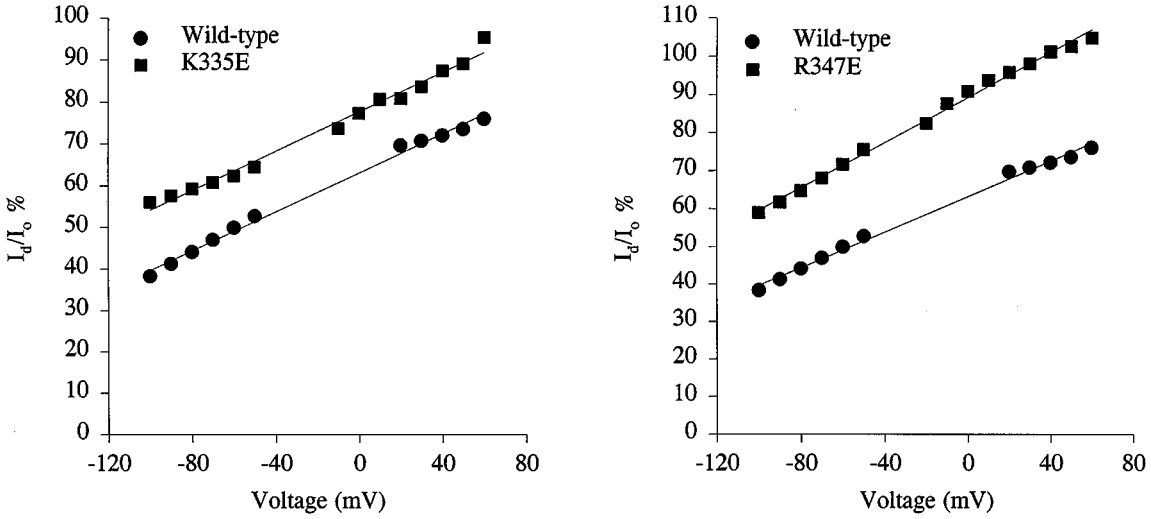

Figure 7 Voltage-dependence of NPPB block of CFTR channels. Relationship between the fractional drug block (I_d/I_0) and the membrane potential determined for the wild-type (100 μM NPPB), K335E (400 μM NPPB) and R347E (1 mM NPPB) CFTR channels. The slopes of the straight lines had values of 0.23 (wild-type), 0.24 (K335E) and 0.30 (R347E).

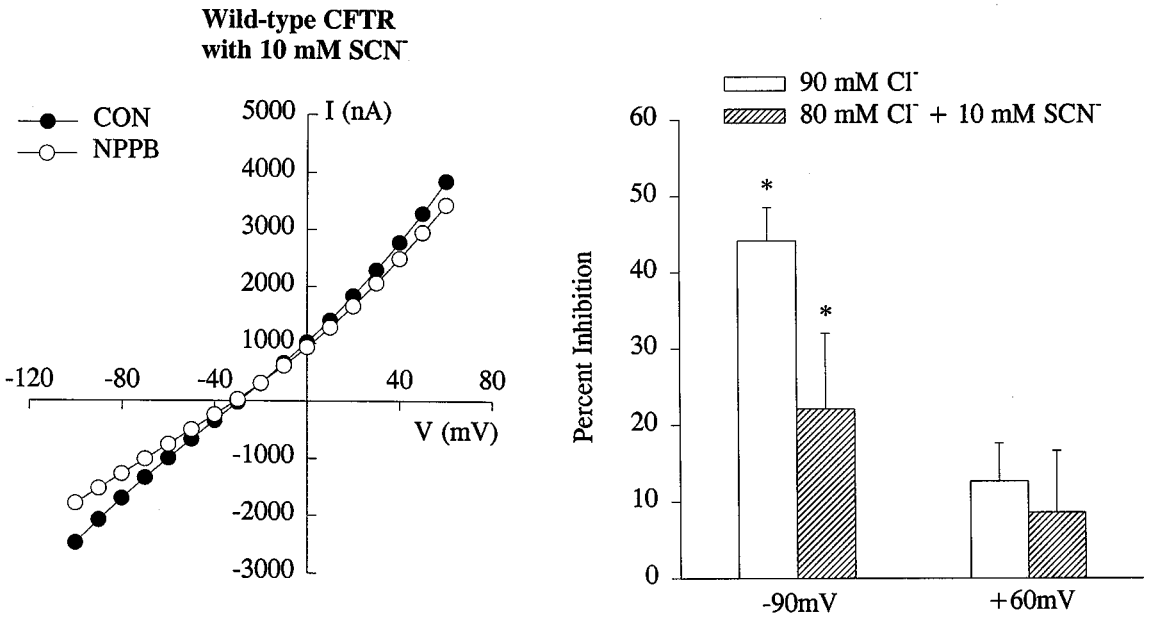

Figure 8 Reduction in NPPB block of the wild-type CFTR channel by SCN^-. Left panel: I/V relationship for the CFTR current measured in the presence and absence of 100 μM NPPB in a bathing solution containing 76 mM NaCl and 10 mM NaSCN. In this experiment NPPB caused decreases of 25 and 10% in the current amplitude measured at -90 and $+60$ mV, respectively. Oocyte O8M27. Right panel: summary of inhibitory effects of 100 μM NPPB on the wild-type CFTR channel measured in normal and NaSCN-containing solutions. Each bar represents the mean$\pm$s.e.mean of five experiments. *($P<0.05$).

Discussion

NPPB block of the wild-type CFTR channel

One goal of this study was to identify structural components of the arylaminobenzoate compound NPPB that are necessary for block of the CFTR Cl^- channel. While previous studies have examined the action of diphenylamine-2 carboxylate (DPC) (McCarty *et al*., 1993; McDonough *et al*., 1994) and glibenclamide (Sheppard & Welsh, 1992; Sheppard & Robinson, 1997) on the CFTR channel, the chemical moieties involved in this block have not been identified. This information is critical if high affinity ligands to the CFTR channel are to be developed.

Several pieces of evidence were obtained that support the view that arylaminobenzoates bind within the pore of the CFTR channel including; the voltage-dependence of NPPB block, the reduction of NPPB block in the presence of the permeant anion SCN^-, and the finding that mutations in the putative pore region of the channel decrease NPPB potency. All of the compounds that were effective in blocking the CFTR channel in this study acted in a similar voltage-dependent manner. McCarty *et al*. (1993) found that DPC and the structurally related chemical, flufenamic acid (at 200 μM concentrations) produce voltage-dependent block of the CFTR current ($\theta \approx 41\%$). Voltage-dependent block of the CFTR channel has also been reported for the stilbene compounds 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS) ($z' = 34$) and 4,4′ diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) ($z' = 16$) (Linsdell & Hanrahan, 1996a), as well as glibenclamide ($\theta = 48\%$) (Sheppard & Robinson, 1997), glutamate ($\theta = 31\%$) and gluconate ($\theta = 30\%$) (Linsdell & Hanrahan, 1996b).

Modifications in the structure of NPPB altered the ability of the drug molecules to block the CFTR channel. The absence of the electron withdrawing nitro group on the benzoate ring, in the compound 2-(3-phenylpropylamino)-benzoic acid, drastically decreased drug potency. Furthermore, removal of the benzoate ring (2-amino-4-phenylbutyric acid) resulted in a complete absence of channel block. Both of these modifications decrease the acidity of the drug molecules by destabilizing the carboxylate anion state. These findings are consistent with previous results showing that compounds with free carboxyl groups, such as clofibric acid, gemfibrozil and chlorophenoxy propionic acid, are relatively ineffective in blocking the CFTR channel (Walsh & Wang, 1996). Our previous studies revealed a strong pH-dependence to NPPB block of the cardiac CFTR isoform and suggested that the ratio of charged to neutral drug molecules determines the degree of current inhibition (Walsh & Wang, 1998). The acidity of the benzoate ring may be another important factor that contributes to drug potency. If this is the case, substitution of acid-strengthening groups (e.g. NO_2, Cl^-, etc.) in the ortho position of NPPB should be expected to enhance channel block when compared with the corresponding meta and para isomers.

While the presence of a benzoate ring in the drug structure was absolutely required for block of the CFTR channel, the absence of the phenyl ring (2-butylamino-5-nitrobenzoic acid) caused only a relatively small change in current block. More importantly, the presence of an additional phenyl ring at the 5′ position (5-nitro-2-(3,3-diphenylpropylamino)-benzoic acid) resulted in enhanced drug potency. These findings are consistent with a model in which the hydrophobic phenyl ring promotes drug interactions with the lipid membrane. Increasing the alkyl chain length of tetraethylammonium derivatives, presumably by enhancing hydrophobic interactions, increases the block of Shaker K^+ channels (Choi *et al*., 1993). Production of new arylaminobenzoate compounds containing substitutions at the phenyl ring end of the NPPB molecule may serve as a fruitful source of more potent CFTR channel blockers.

NPPB block of the mutant CFTR channels

Site-directed mutagenesis experiments have demonstrated that the sixth membrane spanning (M6) segment functions as a major component of the pore-forming region of CFTR. These studies have focused primarily on positively charged lysine and arginine residues found in this segment. Mutations at positions K335 and R347 alter the permeability and conductance ratios of the channel for Cl^- and I^- (Tabcharani *et al*., 1997; Mansoura *et al*., 1998). Furthermore, the R347D construct lacks multi-ion pore behaviour; a property measured with the wild-type channel in mixtures of Cl^- and SCN^- (Tabcharani *et al*., 1993; Linsdell *et al*., 1997). Unlike the wild-type channel, the R347D mutant is insensitive to block by high concentrations of internally applied 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) (Linsdell & Hanrahan, 1996a). Cysteine substitution experiments have also demonstrated that residues R347 and R352 are important in determining the anion versus cation selectivity of charged methanethiosulfonate reagents (Cheung & Akabas, 1996; 1997). We have shown that mutants in the M6 segment display a reduced sensitivity to block by NPPB. This insensitivity is most striking for the R347E mutant, suggesting that this positively charged residue serves as an important determinant in the binding of the negatively charged drug molecules. While the concentration versus response curves for NPPB block of mutants K335E and R347E were shifted to higher concentrations, NPPB produced a voltage-dependent block in the mutants that was almost identical to that of the wild-type channel. This implies that the voltage-dependent nature of NPPB block arises primarily from the response of the charged drug molecules to the membrane potential, and is not due to electrostatic effects occurring within the pore of the channel.

Woodhull analysis (Woodhull, 1973) suggested that NPPB transverses 24% of the electrical field in reaching a binding site from the internal membrane. Although the electrical potential may not be linearly distributed along the CFTR pore, R347 is located at a position approximately 20% from the internal end of M6 peptide sequence. In contrast, DPC is believed to interact at a site 42% across the electric field (McDonough *et al*., 1994). Based on the finding that the mutant S341A displays a 5 fold reduction in DPC affinity, it was suggested that DPC interacts through the hydroxyl group at this residue (McDonough *et al*., 1994). Although the affinity of the R347E mutant for DPC has not been determined, it is likely that mutations at several sites in the M6 region will effect arylaminobenzoate block. This is supported by our observation that a mutation of K335, a site predicted to reside closer to the external mouth of the channel (Riordan *et al*., 1989), resulted in a small, but significant change in NPPB potency ($IC_{50} = 166$ μM for wild-type and 371 μM for K335E).

Effect of external NaSCN in modulating NPPB block

Previous studies have shown that the permeant anion SCN^- reduces the conductance of the wild-type CFTR channel (Tabcharani *et al*., 1993; Hipper *et al*., 1995; Linsdell *et al*., 1997; Mansoura *et al*., 1998). Interestingly, this effect of SCN^- is absent in the R347D mutant (Tabcharani *et al*., 1993). If

R347 functions as an important site in binding both SCN^- and NPPB, then block of the CFTR channel by NPPB should be reduced in the presence of SCN^-. Partial substitution of Cl^- with SCN^- in the bathing solution was indeed found to decrease the inhibitory effect of NPPB on the currents. It was surprising that SCN^- was selective in preventing NPPB block of the inward CFTR currents, since at these negative membrane potentials intracellular Cl^- would be pushed into and extracellular SCN^- out of the pore. However, both inward and outward CFTR currents are reduced during substitution of Cl^- with SCN^- indicating that SCN^- acts as a permeant blocker from either side of the membrane (Linsdell *et al*., 1997). Voltage-dependent block by the arylaminobenzoates is most apparent with low concentrations of the drugs and becomes less prominent as the drug concentration is increased (Walsh & Wang, 1996, 1998). This may imply that drug block involves both direct effects within the pore of the channel and nonspecific actions outside of the channel. Thus, further study of the interactions of NPPB with permeant anions may provide clues to the mechanisms of voltage versus non-voltage dependent block.

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