

Arylamino benzoate Block of the Cardiac Cyclic AMP-Dependent Chloride Current

KENNETH B. WALSH and CHONGMIN WANG

Department of Pharmacology, University of South Carolina, School of Medicine, Columbia, South Carolina 29208

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ABSTRACT

The cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel has been identified in the cardiac muscle of a number of mammalian species, including humans. The goal of this study was to begin quantifying the structural requirements necessary for arylamino benzoate block of the CFTR channel. The cardiac cAMP-dependent Cl^- current (I_{Cl}) was measured using the whole-cell arrangement of the patch-clamp technique in guinea pig ventricular myocytes during stimulation of protein kinase A with forskolin. At drug concentrations below the IC_{50} value for channel block, reduction of I_{Cl} by the arylamino benzoates occurred in a strongly voltage-dependent manner with preferential inhibition of the inward currents. At higher drug concentrations, block of both the inward and outward I_{Cl} was observed. Increasing the length of the carbon chain between

the benzoate and phenyl rings of the arylamino benzoates resulted in a marked increase in drug block of the channel, with IC_{50} values of 47, 17, and 4 μM for 2-benzylamino-5-nitrobenzoic acid, 5-nitro-2-(2-phenylethylamino)-benzoic acid, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), respectively. Increasing the carbon chain length further with the compound 5-nitro-2-(4-phenylbutylamino)-benzoic acid, caused no additional increase in the potency of drug block ($\text{IC}_{50} = 4 \mu\text{M}$). Inhibition of I_{Cl} by the arylamino benzoates was modulated by the pH of the external solution; increasing the pH from 7.4 to 10.0 greatly weakened NPPB block, whereas decreasing the pH to 6.4 enhanced block. In addition, block of I_{Cl} was observed during intracellular dialysis of NPPB, and this action was not affected by raising the external pH.

Cystic fibrosis is an autosomal-recessive disease that results from mutations in the gene encoding the CFTR (Anderson *et al.*, 1992; Riordan, 1994). In epithelial cells from a variety of tissues, CFTR functions as a PKA-activated Cl^- channel (Kartner *et al.*, 1991; Anderson *et al.*, 1991b). In recent years, a cAMP-dependent Cl^- channel also has been identified in guinea pig (Bahinski *et al.*, 1989; Harvey and Hume, 1989a; Ehara and Ishihara, 1990; Matsuoka *et al.*, 1990), rabbit (Harvey and Hume, 1989b), cat (Zhang *et al.*, 1994), simian (Warth *et al.*, 1996), and human (Warth *et al.*, 1996) cardiac ventricular myocytes. The whole-cell and single-channel current properties of the cardiac channel display strong similarity to those of the epithelial CFTR channel (Anderson *et al.*, 1992; Riordan, 1994). Furthermore, with the exception of a deletion in 30 amino acids coded by exon 5 of the CFTR gene, the rabbit cardiac gene shares >90% homology with CFTR (Horowitz *et al.*, 1993; Hart *et al.*, 1996). Expression of the cloned cardiac CFTR channel in *Xenopus*

laevis oocytes results in the appearance of PKA-activated Cl^- currents (Hart *et al.*, 1996). Thus, cardiac tissue expresses an alternatively spliced variant of the CFTR channel.

Ion channel modulators, such as dihydropyridine Ca^{2+} channel antagonists and quaternary ammonium K^+ channel blockers, have proved useful in elucidating channel gating mechanisms and mapping ion-permeation pathways. Although the CFTR Cl^- channel has been widely studied in both human epithelial tissues and heterologous cells expressing the CFTR gene (Anderson *et al.*, 1992; Riordan, 1994), little quantitative data are available concerning the pharmacology of this channel. Previous studies of CFTR have focused on the compound DPC, which blocks the CFTR channel when applied at relatively high concentrations (200 μM to 3 mM) (Anderson *et al.*, 1991b, 1992; McCarty *et al.*, 1993). Greger and colleagues modified the structure of DPC to produce a group of arylamino benzoate compounds that varied in the phenyl-to-benzoate group, carbon chain length (Wangemann *et al.*, 1986). One of these arylamino benzoates, NPPB, was identified as a potent blocker of Cl^- channels in the TAL of the kidney (Wangemann *et al.*, 1986; Tilmann *et al.*, 1991). However, the effects of NPPB and other arylamino benzoates

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ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; I_{Cl} , cAMP-dependent Cl^- current; BNBA, 2-benzylamino-5-nitrobenzoic acid; NPEB, 5-nitro-2-(2-phenylethylamino)-benzoic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; NPBA, 5-nitro-2-(4-phenylbutylamino)-benzoic acid; DMSO, dimethylsulfoxide; DPC, diphenylamine-2-carboxylate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

have not been studied in detail on the CFTR Cl^- channel. Thus, the goal of the current study was to quantify the blocking action of arylaminobenzoates on the cardiac cAMP-dependent Cl^- current.

Materials and Methods

Isolation of cardiac myocytes. An enzymatic dissociation procedure based on that of Mitra and Morad (1985) was used to isolate the myocytes. Briefly, hearts were removed from adult guinea pigs (200–300 g), mounted on a Langendorf-type column, and perfused for 10 min with a Ca^{2+} -free Tyrode's solution containing collagenase (0.25–0.32 units/ml) (type B; Boehringer-Mannheim Biochemicals, Mannheim, Germany) and protease (0.2 mg/ml) (type 14 or 25; Sigma Chemical, St. Louis, MO). After 10 min of perfusion with 0.2 mM Ca^{2+} -containing Tyrode's, the heart was dissected into small pieces and single cells obtained by gentle agitation. Cells were stored at room temperature (22–25°) in normal Tyrode's solution (see below) and used 1–10 hr after isolation.

Recording procedure. The patch-clamp method (Hamill *et al.*, 1981) was used to record whole-cell ventricular currents using PC-501 (Warner Instrument, Hamden, CT), L/M EPC-7 (Adams and List, Westbury, NY), and Axopatch 200 (Axon Instruments, Foster City, CA) amplifiers. Pipettes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams, Parsippany, NJ) and had resistances of 1–3 M Ω when filled with internal solution. Series resistance was determined by measuring the time constant of the capacity current and the membrane capacitance. For a typical set of experiments, series resistance ranged from 5.2 to 7.9 M Ω in the guinea pig ventricular myocytes with a mean \pm standard error of 6.2 ± 0.2 M Ω (18 cells). Typically, > 50% of the series resistance could be compensated electronically. Membrane currents were measured with 12-bit analog/digital converters (Scientific Associates and Axon Instruments). Data were sampled at 10 KHz, filtered at 2–3 KHz with a low-pass Bessel filter (Frequency Devices, Haverhill, MA), and stored using personal computers [Northgate (Edn Prairie, MN) and Dell (Austin, TX)].

A reference electrode made from an Ag/AgCl pellet was connected to the bath using an agar salt bridge saturated with Tyrode's solution. Data were adjusted for liquid junction potentials that arose both between the pipette solution and bath solution and between the reference electrode and the bath (Walsh and Long, 1994). Liquid junction potential values were measured at the start and end of experiments and were between 0 and +5 mV.

Measurement of the cardiac I_{Cl} . Isolated cells were initially placed in a normal Tyrode's solution consisting of 132 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 5 mM dextrose, and 5 mM HEPES, pH 7.4. After establishment of the whole-cell voltage-clamp, the solution was changed to a K^+ -free solution containing 140 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM dextrose, 5 mM HEPES, and 1 mM BaCl_2 , pH 7.4 (total $[\text{Cl}^-] = 146$ mM) (osmolarity, 280 mOsm). I_{Ca} was eliminated by the addition of 200–500 nM nisoldipine (Miles Laboratories, West Haven, CT) to the external solution. I_{Na} was eliminated by holding the membrane potential at -40 mV and adding 10 μM tetrodotoxin to the bath. Patch electrodes were filled with a pipette solution consisting of 70 mM CsCl, 40 mM Cs-aspartate, 2 mM MgCl_2 , 1 mM CaCl_2 , 11 mM EGTA, 5 mM ATP (K^+ salt), and 10 mM HEPES, pH 7.3 (total $[\text{Cl}^-] = 76$ mM) (osmolarity, 280 mOsm). The ratio of EGTA/ CaCl_2 in these solutions sets the free intracellular Ca^{2+} concentration to ~ 10 nM (Fabiato, 1988).

The cardiac I_{Cl} was recorded during voltage steps applied to various potentials from a holding potential of -40 mV. A small recording chamber (0.5-ml volume) was used to facilitate solution changes. I_{Cl} was activated by the addition of 2 μM forskolin to the external solution, and drug block was quantified after 5 min of arylaminobenzoate exposure. This represents a time point at which block of I_{Cl} had saturated (see Fig. 4). To determine possible current rundown

during these experiments, control measurements were performed over this time period. At +60 mV, there was no rundown in I_{Cl} observed during the first 5–6 min of experimentation. In contrast, at -90 mV, there was a decrease of $8 \pm 5\%$ (10 cells) in I_{Cl} . Because of the variability in current amplitude from one cell to another, we did not correct for this decrease in the drug experiments.

Expression of CFTR in *X. laevis* oocytes. The cDNA for the human epithelial CFTR channel was generously supplied by Dr. Alan Smith (Genzyme, Cambridge, MA), and CFTR transcripts were prepared using the mMessage mMachine kit (Ambion, Austin, TX). Stage V and VI oocytes were injected with 50 nl of cRNA (0.1–0.2 mg/ml) using a microinjector (Drummond Scientific, Broomall, PA). CFTR Cl^- currents were measured 1–3 days after injection using a TEV 200 two microelectrode voltage clamp (Dagan, Minneapolis, MN). The oocyte bathing solution consisted of 96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , and 5 mM HEPES, pH 7.4. CFTR currents were activated by application of a cAMP cocktail containing forskolin (10 μM), 8-chlorophenylthio cAMP (500 μM) and 3-isobutyl 1-methylxanthine (1 mM).

Preparation and use of the arylaminobenzoate compounds. The structure of the drugs BNBA, NPEB, NPPB, and NPBA are shown in Fig. 1. These arylaminobenzoates were generously supplied by Dr. Rainer Greger (Albert-Ludwigs University, Freiburg, Germany). The drugs differ in the length of the carbon chain between the benzoate (with carboxyl group) and phenyl rings. The pK_a of NPPB was determined titrimetrically to be ~ 4.5 . This is consistent with a previous reported calculation (Wangemann *et al.*, 1986). The percentage of charged drug (C) was calculated using the equation:

$$C = K_a / (K_a + [\text{H}]_0) \quad (1)$$

where K_a is the acid dissociation constant for the drug molecule. Stock solutions of the drugs were prepared in 100% DMSO and diluted into the external solution so the final volume of DMSO was $\leq 0.1\%$.

The voltage dependence of drug block was determined by fitting the relationship between the $K_d(\text{V})$ of the compound [$K_d(\text{V}) = [\text{drug}] * \text{I}_d/\text{I}_o - \text{I}_d$, with I_o and I_d representing the I_{Cl} amplitudes measured before and after the addition of the drug, respectively] and the voltage with the equation:

$$K_d(\text{V}) = K_d(0) * \exp(z\theta\text{FV}/\text{RT}) \quad (2)$$

where $K_d(0)$ is the K_d value 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 the valence (z) was -1 and there was a single binding site for the arylaminobenzoates.

All experiments were conducted at room temperature (22–25°). Averaged values presented are mean \pm standard error. Where ap-

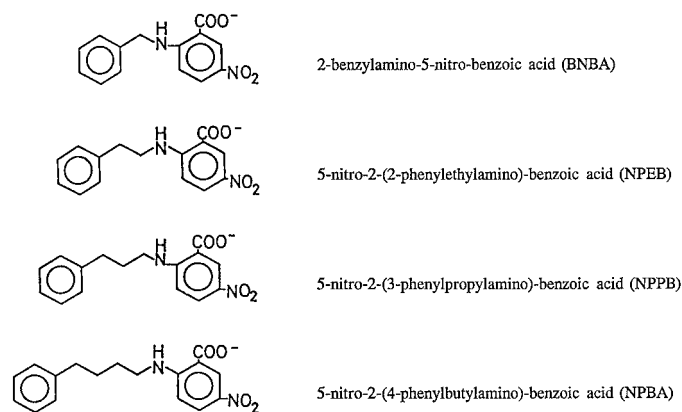


Fig. 1. Structure of arylaminobenzoates used in this study.

appropriate, statistical significance was estimated using Student's *t* test for unpaired observations.

Results

Arylamino benzoate block of the cardiac cAMP-dependent Cl^- channel. Fig. 2 (*top*) displays whole-cell background currents recorded from a cardiac ventricular cell during 100-msec voltage steps applied to various potentials. The external application of $2\ \mu\text{M}$ forskolin caused the activation of a time-independent, outward-rectifying Cl^- current when recorded with a Cl^- concentration of $76\ \text{mM}$ in the pipette and $146\ \text{mM}$ in the bath ($E_{\text{Cl}} = -16\ \text{mV}$). The physiological properties of this cardiac I_{Cl} have been described previously (Bahinski *et al.*, 1989; Harvey and Hume, 1989a; Matsuoka *et al.*, 1990; Walsh and Long, 1992). As shown in Fig. 2, the addition of a $20\ \mu\text{M}$ concentration of NPPB caused a strong reduction in both inward and outward I_{Cl} . Overall in six myocytes examined, $20\ \mu\text{M}$ NPPB decreased the inward (at $-90\ \text{mV}$) and outward (at $+60\ \text{mV}$) I_{Cl} by $94 \pm 4\%$ and $91 \pm 4\%$, respectively. Partial recovery from NPPB block could be observed during long periods of drug washout (5–10 min). In three washout experiments, I_{Cl} recovered to within $16 \pm 10\%$ of the initial amplitude measured in the absence of the drug. A more rapid and complete recovery from drug block was

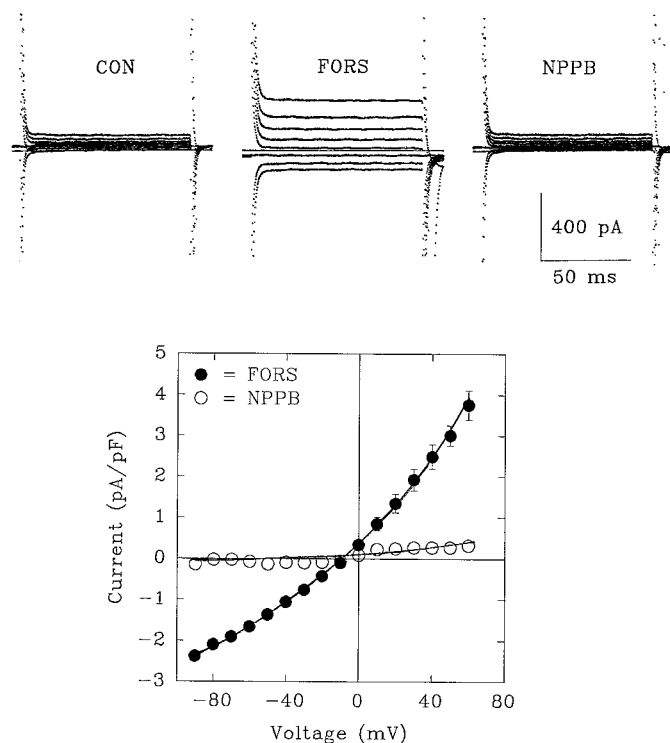


Fig. 2. Block of the cardiac I_{Cl} by NPPB. *Top*, whole-cell background currents recorded during 100-msec voltage steps applied from a holding potential of $-40\ \text{mV}$ to $-80\ \text{mV}$ through $+60\ \text{mV}$ in 20-mV increments. Currents were measured under basal conditions (CON) and after the addition of $2\ \mu\text{M}$ forskolin (FORS). The forskolin-activated I_{Cl} was inhibited by 100% and 96%, respectively, at -80 and $+60\ \text{mV}$ by a $20\ \mu\text{M}$ concentration of NPPB. Cell AB4-368. *Bottom*, current-voltage relationship for forskolin-sensitive I_{Cl} obtained in the presence and absence of $20\ \mu\text{M}$ NPPB. Values are mean \pm standard error for six experiments. Values for NPPB were obtained at 5 min after addition of the drug to the bath; this represents a time period when block of I_{Cl} by NPPB had saturated (see Fig. 4).

obtained in experiments with lower concentrations of NPPB (see Fig. 9).

Voltage-dependence of arylamino benzoate block. The expressed epithelial CFTR Cl^- channel is blocked by the arylamino benzoate DPC ($200\ \mu\text{M}$) in a strongly voltage-dependent manner (McCarty *et al.*, 1993; McDonough *et al.*, 1994). Because voltage-dependent block of the cardiac channel was not evident in the initial experiments with $20\ \mu\text{M}$ NPPB (Fig. 2), it was determined whether voltage-dependent block might be observed with lower concentrations of the drug. The application of $2\ \mu\text{M}$ NPPB produced a clear voltage-dependent block of the channels with $> 30\%$ reduction in the inward current and no decrease in the outward current (Fig. 3). The electrical distance sensed by the NPPB molecule, θ , was determined using Woodhull analysis (Woodhull, 1973) as described in Materials and Methods. An average θ of $32 \pm 3\%$ (four myocytes) was calculated with the orientation from the inside membrane. A similar value ($\theta = 28 \pm 5\%$) was measured during internal dialysis of NPPB. Relatively low concentrations of other arylamino benzoates also blocked I_{Cl} in a voltage-dependent manner. For example, the drug BNBA ($25\ \mu\text{M}$) blocked the inward current (at $-90\ \text{mV}$) by $34 \pm 7\%$ but inhibited the outward current (at $+60\ \text{mV}$) by only $4 \pm 4\%$. In four experiments, an average θ of $31 \pm 2\%$ was determined with this drug concentration.

Voltage-dependent block of the cardiac I_{Cl} also was apparent immediately after the application of high concentrations of the drugs. Fig. 4 plots the time course of NPPB ($20\ \mu\text{M}$) block of I_{Cl} , measured during voltage steps to either -90 and $+60\ \text{mV}$. As shown in Fig. 4 (*left*), there was a strong block of the inward I_{Cl} during the initial 30 sec after NPPB addition to the chamber but no block of the outward I_{Cl} during this time. However, both inward and outward currents were reduced to a similar extent after 4–5 min of drug exposure. In three time course experiments, the inward current and outward currents were reduced by $32 \pm 13\%$ and $0 \pm 2\%$, respectively, after a 30-sec exposure to NPPB.

Effect of increasing carbon chain length on arylamino benzoate potency. Previous studies have shown that increasing the carbon chain length between the benzoate and phenyl rings of the arylamino benzoates increases the potency for Cl^- current inhibition in the thick ascending limb of the kidney (Wangemann *et al.*, 1986). To determine whether a similar order of potency exists for block of the cardiac cAMP-dependent channel, we tested the effect on I_{Cl} of the drugs shown in Fig. 1. Fig. 5 displays concentration-versus-inhibition curves for the drugs BNBA, NPEB, NPPB, and NPBA. Because block of I_{Cl} by the arylamino benzoates was voltage dependent, especially at low drug concentrations (see Fig. 3), all the data displayed in Fig. 5 were recorded at $-90\ \text{mV}$. Increasing the carbon chain length produced a clear increase in the potency of arylamino benzoate block of the cardiac cAMP-dependent channel, with IC_{50} values of $47\ \mu\text{M}$, $17\ \mu\text{M}$, and $4\ \mu\text{M}$ for BNBA (one carbon atom), NPEB (two carbon atoms), and NPPB (three carbon atoms), respectively. Increasing the carbon chain further with NPBA (four carbon atoms) caused no additional increase in drug potency over that of NPPB (IC_{50} for NPBA = $4\ \mu\text{M}$).

pH dependence of arylamino benzoate block. All of the drugs shown in Fig. 1 contain a negatively charged carboxyl group that causes the pK_a of the compounds to be < 5 . The pK_a of NPPB was determined to be close to 4.5, and thus

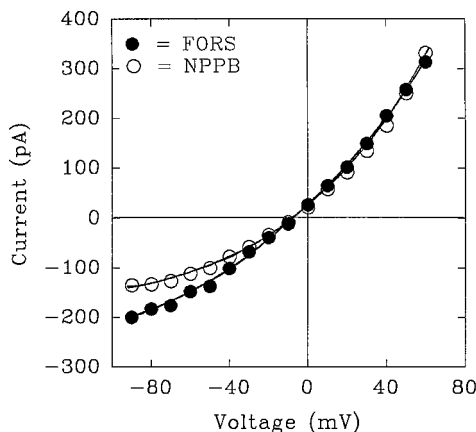
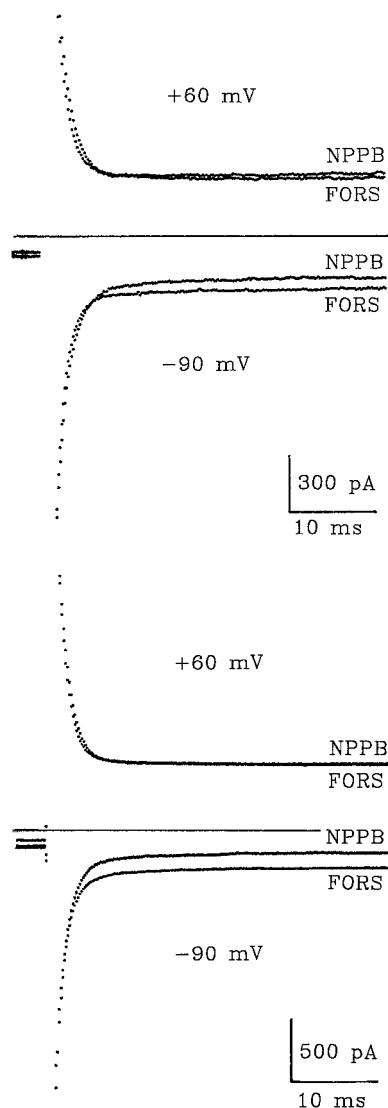


Fig. 3. Voltage-dependent block of the cardiac I_{Cl} by NPPB. *Left*, forskolin (FORS)-activated I_{Cl} recorded during 100-msec voltage steps applied to either -90 or $+60$ mV in the presence and absence of $2 \mu\text{M}$ NPPB. I_{Cl} was inhibited by 32% and 0%, respectively, at -90 and $+60$ mV. *Right*, current-voltage relationship for I_{Cl} shown on the *left*. Cell AB4-C08.

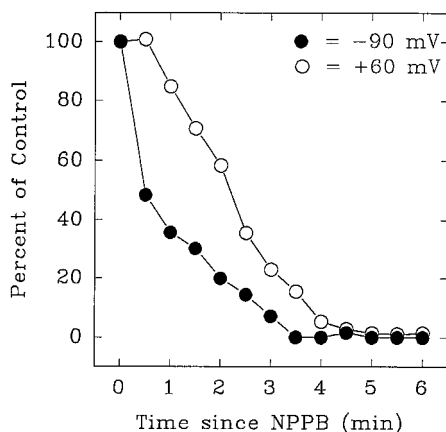
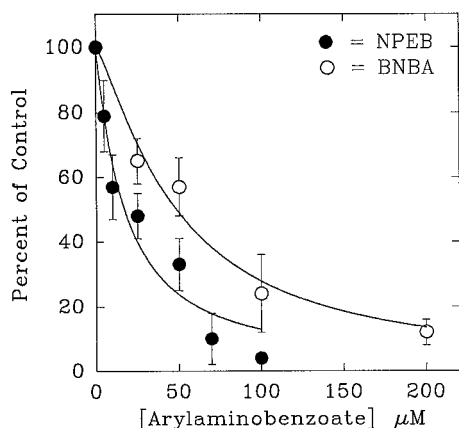


Fig. 4. Time course of NPPB inhibition of the cardiac I_{Cl} . *Left*, forskolin-activated I_{Cl} recorded during 100-msec voltage steps applied to either -90 or $+60$ mV both before (FORS) and 30 sec after the addition of $20 \mu\text{M}$ NPPB. *Right*, time course for inhibition of I_{Cl} by NPPB measured at either -90 or $+60$ mV. Percentage inhibition is relative to that obtained just before NPPB addition (time = 0). Cell AB4-452. In separate experiments, the solution exchange "dead space" for the chamber used in these experiments was determined by measuring the block of the inward rectifier K^+ current by 1 mM BaCl_2 . The "dead space" value was 2–10 sec.

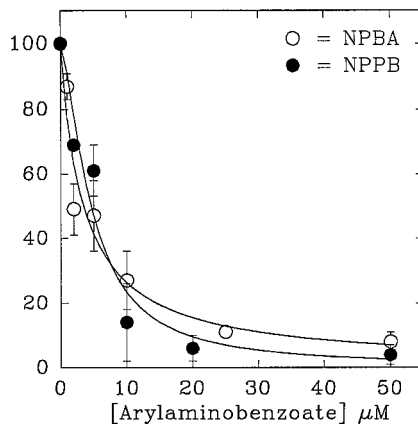


Fig. 5. Concentration-versus-response curves for inhibition of I_{Cl} by the arylamino benzoates. Values are mean \pm standard error of three to six experiments. The theoretical curves for the data are given by $[\text{drug}]/(\text{IC}_{50} + [\text{drug}])$ with IC_{50} values of 47, 17, 4, and $4 \mu\text{M}$ for BNBA, NPEB, NPBA, and NPPB, respectively, providing the best least-squares fit to the points.

according to eq. 1, the drug molecules are predominately charged ($>99\%$) in external solution at pH 7.4. Increasing the pH of the solution to 10.0 should not significantly alter the amount of charged to neutral form of the drug (100% of NPPB molecules are ionized at pH 10.0). It was surprising to find, therefore, that block of the cardiac I_{Cl} by NPPB was almost completely abolished when the pH of the external solution

was increased to 10.0 (Fig. 6). Even at an NPPB concentration of $50 \mu\text{M}$, which produces $>95\%$ block at pH 7.4, there was only a small and nonsignificant decrease in the current at pH 10.0 ($10 \pm 6\%$, four cells, $p > 0.2$). However, a strong and rapid block of the current could be induced at this time by replacing the external solution with a drug-containing external solution buffered to pH 7.4 (results not shown), as

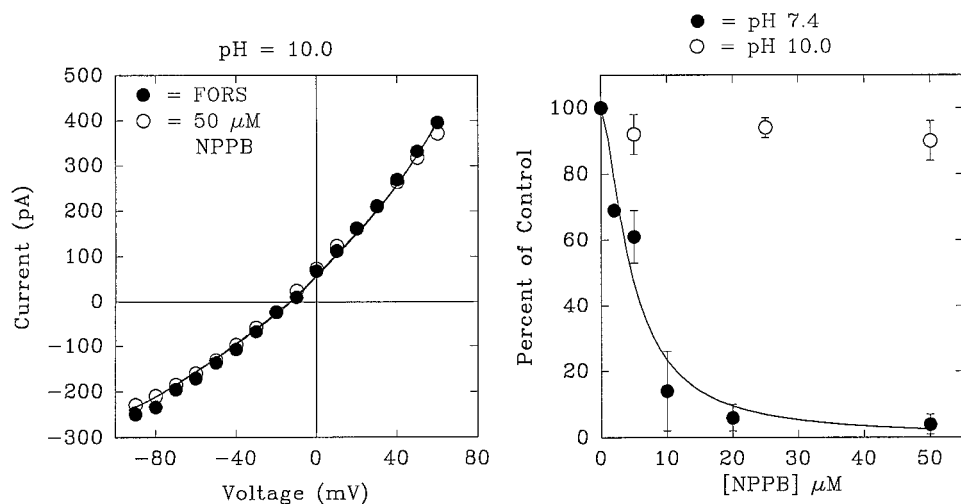


Fig. 6. Increasing extracellular pH prevents the block of I_{Cl} by NPPB. *Left*, current-voltage relationship for I_{Cl} measured in the presence and absence of 50 μ M NPPB in external solution at pH 10.0. Cell AB4-606. FORS, forskolin. *Right*, concentration-versus-response curve for inhibition of I_{Cl} by NPPB. Data at pH 7.4 are taken from Fig. 5. For the data at pH 10.0, values are mean \pm standard error of three or four experiments.

expected from the previous experiments (see Fig. 2). Increasing the internal solution pH also decreased the block of the I_{Cl} by NPPB. At a concentration of 20 μ M, NPPB caused a $94 \pm 4\%$ ($n = 4$) decrease in the current at pH 7.3 and $61 \pm 6\%$ (five cells) decrease at pH 8.4. This difference was statistically significant ($p < 0.05$).

Because raising the pH of the external solution to 10.0 greatly weakened the block of the cardiac I_{Cl} by NPPB, it was determined whether lowering the external pH also might influence drug action. According to eq. 1, $\sim 1.2\%$ of the drug molecules are neutral at pH 6.4. As shown in Fig. 7, decreasing the pH of the external solution to 6.4 resulted in an enhanced block of I_{Cl} by NPPB with the IC_{50} value decreasing from 4 μ M at 7.4 to 0.6 μ M at 6.4. Under these conditions, a substantial block of I_{Cl} ($35 \pm 11\%$, three cells) could be obtained with a NPPB concentration as low as 500 nM. As was the case at pH 7.4, block of I_{Cl} occurred in a voltage-dependent manner.

Internal block by NPPB. The results displayed in Figs. 2-7 indicate that arylamino benzoate block of the cardiac cAMP-dependent Cl^- channel occurs preferentially at negative membrane potentials and is enhanced by lowering the pH of the external solution. One explanation for these results would be that the drugs act primarily through a membrane-accessible pathway. Decreasing the external pH might en-

hance the movement of the drugs into the membrane by altering charged groups on the drug. Once inside the cell, block by the charged drug molecules (at the internal pH 7.3) would be favored at negative membrane potentials. To test this model, NPPB was added to the patch pipettes and dialyzed into the myocytes.

Fig. 8 summarizes the results of experiments in which 10 μ M NPPB was dialyzed into the ventricular cells. For the data displayed in Fig. 8 (*left*), the cells were bathed in external solution buffered to pH 7.4. The results (Fig. 8, *right*) were obtained in external solution at pH 10.0. In each experiment, NPPB was allowed to dialyze into the myocyte for 10-12 min before the application of forskolin to activate I_{Cl} . Based on previous dialysis experiments and the parameters of the current study (i.e., myocyte size, electrode resistance, and so on) (Pusch and Neher, 1988; Walsh and Long, 1994), it was predicted that NPPB should equilibrate between the pipette and interior of the cell with a time constant of 3-4 min. Internal NPPB produced a strong block of both the inward and outward Cl^- currents compared with DMSO-dialyzed, control myocytes (Fig. 8, FORS). In five experiments, NPPB reduced the average current measured at -90 mV by 89%. This is similar to that obtained with external NPPB ($85 \pm 10\%$). Most importantly, during internal dialy-

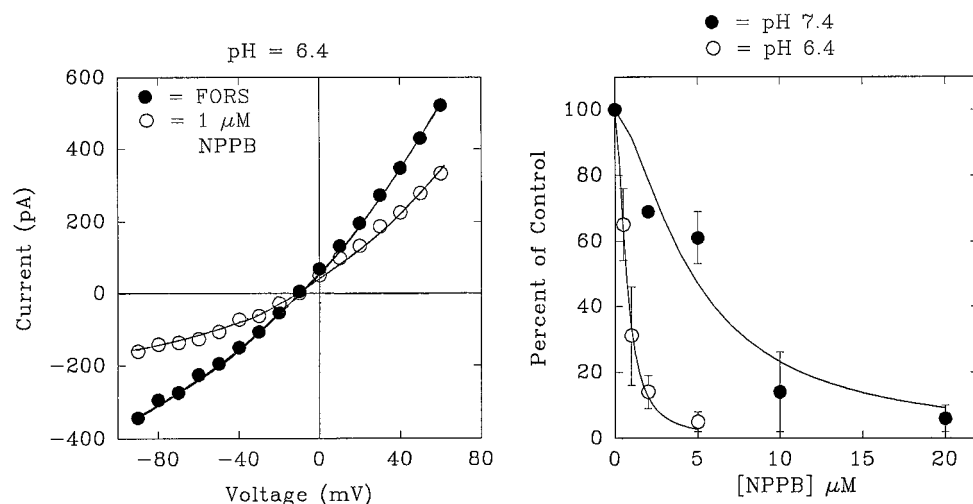


Fig. 7. Decreasing extracellular pH augments the block of I_{Cl} by NPPB. *Left*, current-voltage relationship for I_{Cl} measured in the presence and absence of 1 μ M NPPB in external solution at pH 6.4. Cell AB4-651. FORS, forskolin. *Right*, concentration-versus-response curve for inhibition of I_{Cl} by NPPB. Data at pH 7.4 are taken from Fig. 5. For the data at pH 6.4, values are mean \pm standard error of three to five experiments. The theoretical curve for the data at pH 6.4 provided an IC_{50} value of 0.6 μ M.

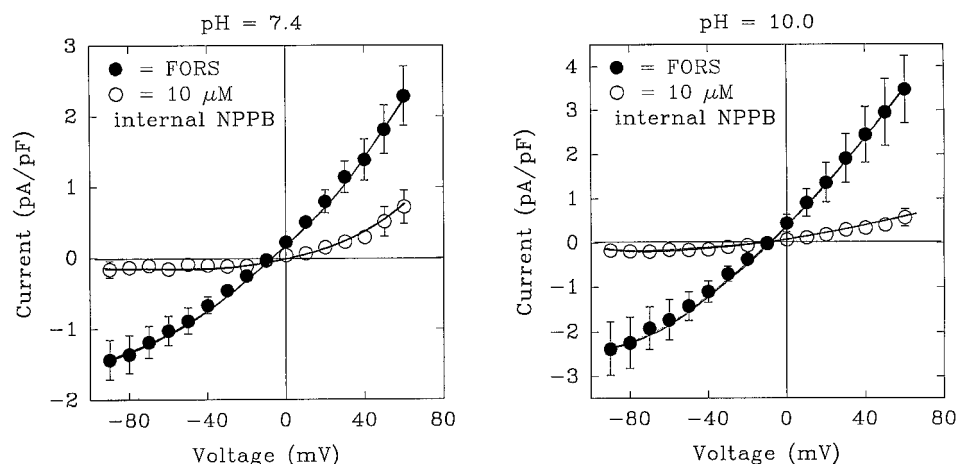


Fig. 8. Block of I_{Cl} by internally applied NPPB. Current-voltage relationships for I_{Cl} measured during dialysis with internal solution containing DMSO (FORS) or 10 μ M NPPB. In each experiment, a 10–12-min period was allowed for dialysis after breakthrough into the whole-cell configuration. After this dialysis period, the cells were exposed to 2 μ M forskolin, and the peak current was measured. Results were obtained in external solution at pH 7.4 (left) or 10.0 (right). Values are mean \pm standard error of three or four experiments. There was a significant difference ($p < 0.02$) between the control and NPPB data. In contrast, there was no significant difference ($p > 0.5$) between the NPPB values shown on the left and right.

sis, increasing the external pH to 10.0 did not prevent NPPB block of either the inward or outward currents (Fig. 8, right).

Drug recovery experiments also were performed to gain further information on the mechanism of NPPB block. For these experiments, I_{Cl} was first blocked by NPPB at pH 7.4. The drug then was washed out of the chamber with external solution at either pH 7.4 or 10.0. If NPPB binds to a pH-regulated site on the external side of the cell membrane, the rate of recovery from drug block at pH 10.0 should be significantly faster than that obtained at pH 7.4. In contrast, if NPPB acts through a membrane-accessible pathway, screened from the external solution, the recovery at the two pH values should not be significantly different. As shown in Fig. 9, the time course of recovery from NPPB (5 μ M) block at -90 mV was similar at both pH values. In three experiments each, the time constant for recovery was 3.5 ± 0.4 min at pH 7.4 and 2.9 ± 0.3 min at pH 10.0 ($p > 0.05$).

NPPB block of the epithelial CFTR channel. NPPB was found to block the cardiac I_{Cl} in a voltage- and pH-dependent manner (Figs. 3, 6, and 7). Because the cardiac I_{Cl} is believed to represent an alternatively spliced variant of the CFTR Cl^- channel (Horowitz *et al.*, 1993; Hart *et al.*, 1996), it was important to determine whether NPPB has similar actions on the epithelial form of the channel. Fig. 10 displays the results of experiments in which the effect of NPPB was determined on the human epithelial CFTR channel expressed in *X. laevis* oocytes. At a concentration of 50 μ M, externally applied NPPB caused a voltage-dependent block of the CFTR current (Fig. 10, left) with a $25 \pm 2\%$ decrease

measured at -90 mV, and a $3 \pm 2\%$ change at $+60$ mV (four oocytes). As was the case with the cardiac I_{Cl} , block of the CFTR current could be abolished completely when the pH of the external solution was increased to pH 10 (Fig. 10, right) (percentage change at -90 and $+60$ mV = $1 \pm 3\%$ and $6 \pm 2\%$, respectively; five oocytes).

Discussion

Arylamino benzoate block of the cardiac cAMP-dependent channel. The goal of the current study was to begin quantifying the structural requirements necessary for drug block of the cardiac cAMP-dependent Cl^- channel. This was accomplished by studying the effect of a group of arylamino benzoate compounds that varied in the phenyl-to-benzoate ring, carbon chain length. Our approach was based on previous findings that the arylamino benzoate compound DPC blocks both the epithelial (Anderson *et al.*, 1992; McCarty *et al.*, 1993) and cardiac (Walsh and Wang, 1996) isoforms of the CFTR channel. McCarty *et al.* (1993) found that DPC and the structurally related chemical flufenamic acid (at 200 μ M concentrations) produce a $\sim 40\%$ block of the CFTR Cl^- current in *X. laevis* oocytes expressing the human epithelial channel. Single-channel analysis suggested that DPC is an open channel blocker of CFTR that permeates through the membrane to reach its binding site (McCarty *et al.*, 1993). In guinea pig ventricular myocytes, DPC reduces the whole-cell I_{Cl} with an IC_{50} value of 270 μ M (Walsh and Wang, 1996).

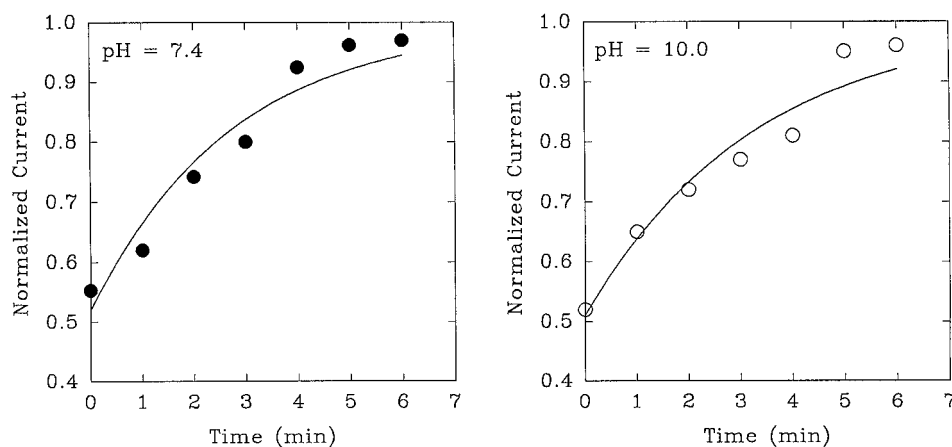


Fig. 9. Influence of external pH on the recovery of I_{Cl} block by NPPB. I_{Cl} was blocked by 5 μ M NPPB at pH 7.4, and the time course of recovery determined during washout with external solution at pH 7.4 (left) and 10.0 (right). In each experiment, the currents recorded at -90 mV were normalized to the control data measured before NPPB application. Time = 0 represents the last point taken before starting NPPB washout. The data were fit with a single exponential function with time constants of 2.9 (pH 7.4) and 3.3 (pH 10.0) min. Cells AB4–841 and AB4–808.

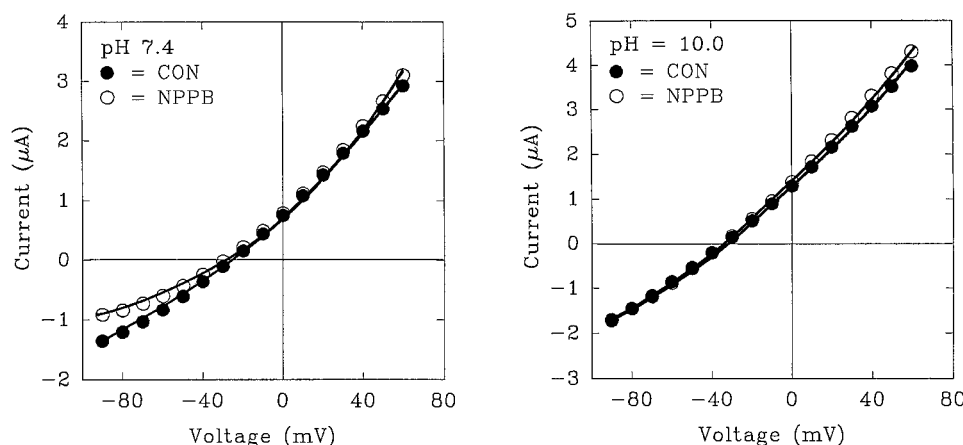


Fig. 10. Block of the human epithelial CFTR Cl^- channel by NPPB. *Left*, current-voltage relationship for CFTR current expressed in a *X. laevis* oocyte and measured in the presence and absence of 50 μM NPPB in external solution at pH 7.4. In this experiment, NPPB caused a 30% decrease in the current measured at -90 mV. Oocyte 8202. CON, control. *Right*, current-voltage relationship for the CFTR current measured in the presence and absence of 50 μM NPPB in external solution at pH 10.0. No change was recorded in the current measured at -90 mV. Oocyte 8222.

The most significant finding of this study was that at least up to the compound NPBA, there was no exclusion of any of the arylaminobenzoates from the cardiac Cl^- channel binding site. In fact, there was an overall tendency for increased potency with increased drug size (see Fig. 5). The incorporation of an extra carbon chain between the amino group and phenyl ring of DPC, to produce BNBA, resulted in a 6-fold increase in the potency of I_{Cl} block ($\text{IC}_{50} = 47$ μM for BNBA) over that for DPC (Walsh and Wang, 1996). Increasing the carbon chain length beyond that of BNBA in the drugs NPEB and NPPB caused a sequential decrease in the IC_{50} for current block ($\text{IC}_{50} = 17$ and 4 μM for NPEB and NPPB, respectively). These results display a striking similarity to the block of squid axon (Armstrong, 1971) and *Drosophila* Shaker (Choi *et al.*, 1993) K^+ channels by tetra-*n*-alkylammonium ions, in which increasing the size of the alkyl chain increases drug potency. Thus, as is the case for K^+ channels, block of the cardiac Cl^- channel seems to involve interactions of the lipophilic alkyl chain of the arylaminobenzoates with hydrophobic moieties either on the channel or within the lipid membrane.

Voltage and pH dependence of arylaminobenzoate block. Arylamino benzoate block of the cardiac cAMP-dependent current occurred in both a voltage- and pH-dependent manner. Voltage-dependent block of the epithelial CFTR channel has been reported for DPC and flufenamic acid ($\theta = 41\%$ from the inside) (McCarty *et al.*, 1993), as well as glutamate ($\theta = 31\%$) and gluconate ($\theta = 30\%$) (Linsdell and Hanrahan, 1996). In the current study, voltage-dependent block was most evident using relatively low concentrations of the drugs or immediately after the application of higher concentrations of the compounds. NPPB has length of ~ 16 Å (Wangemann *et al.*, 1986), yet it blocked the I_{Cl} with the same θ ($\sim 30\%$) as BNBA, which has a length of ~ 12 Å. This implies that although these two compounds are of different size, they act at a similar site $\sim 30\%$ within the electric field. This apparent discrepancy can be reconciled if the lipophilic phenyl ring of the compounds penetrates to varying amounts into the lipid membrane (see above). This might allow the negatively charged carboxyl group on each of the arylaminobenzoates to interact at a similar binding site either on or near the channel. For the epithelial CFTR channel, a serine at position 341 represents at least one hypothetical binding site for the carboxyl group of DPC (McDonough *et al.*, 1994). Mutations at this site reduce the potency of DPC block of the

channel (McDonough *et al.*, 1994). If the phenyl ring is involved in docking the compounds within the lipid membrane, substitutions on this ring, which reduce the lipophilicity, may also reduce the drug potency.

Changes in the external pH modulate ion channel block by local anesthetics (Hille, 1977; Schwarz *et al.*, 1977) and dihydropyridine Ca^{2+} channel antagonists (Uehara and Hume, 1985; Kass and Arena, 1989) by altering the ratio of charged to neutral drug forms. One surprising result of this study was that increasing the external pH from 7.4 to 10.0 almost completely eliminated NPPB block of I_{Cl} . With a pK_a of ~ 4.5 , NPPB is in a predominately charged form ($>99\%$) at pH 7.4, and increasing the pH should cause only a slight change in the fraction of charged drug (100%). The results of this study could be explained if channel block occurs after the movement of the neutral form of the drugs into the cell membrane. Once internalized, conversion to the charged form, at the internal pH of 7.3, would promote the voltage-dependent block measured with the compounds. This model implies that relatively low concentrations of the drugs at pH 7.4 can block the cardiac Cl^- channel. Increasing the external pH to 10.0, by removing the neutral form of the drug (essentially 0%), eliminates drug block (Fig. 6). In contrast, decreasing the external pH to 6.4, by increasing the neutral form of the drug (1.2% neutral drug) enhances drug block (Fig. 7). This model is consistent with the results demonstrating that block by internally applied NPPB is not affected by the external pH (Fig. 8), whereas block by externally applied NPPB is regulated by increasing the internal pH.

Changes in the external pH also could have direct actions on the channel protein that modulate drug binding. Increasing the external pH might eliminate NPPB block by titrating positively charged amino acid residues in the Cl^- channel, which interact with the carboxyl group of the drugs. Both a lysine at position 335 and an arginine at position 347 are found in the sixth membrane spanning segment of CFTR and contribute positive charge to the pore of the channel (Anderson *et al.*, 1991a; McDonough *et al.*, 1994). However, this model fails to explain why decreasing the external pH increases drug potency or why increasing the external pH has no effect on I_{Cl} block by internal NPPB (Fig. 8). Furthermore, the presence of an externally modulated drug binding site is not supported by the drug recovery experiments (Fig. 9), in which drug recovery should have been enhanced under alkaline conditions. Therefore, pH-induced change in the charge

of the drug molecules remains the most reasonable explanation for the observed modulatory actions.

Relevance of the study to cardiac pharmacology. A β -adrenergic-activated Cl^- channel has been identified in guinea pig (Bahinski *et al.*, 1989; Harvey and Hume, 1989a; Ehara and Ishihara, 1990; Matsuoka *et al.*, 1990), rabbit (Harvey and Hume, 1989b), cat (Zhang *et al.*, 1994), and simian (Warth *et al.*, 1996) cardiac ventricular cells. Molecular analysis strongly suggests that these cells express an alternatively spliced variant of the CFTR channel (Horowitz *et al.*, 1993; Hart *et al.*, 1996). Although an initial report suggested that CFTR is expressed in human cardiac tissue (Levesque *et al.*, 1992), other studies have failed to identify a cAMP-dependent Cl^- current in human cardiac myocytes (Oz and Sorota, 1995; Sakai *et al.*, 1995). If present in the human heart, the CFTR channel could play an important role in controlling the action potential duration. Due to the properties of the cardiac CFTR current-voltage relationship, activation of this channel during β -adrenergic stimulation will cause a decrease in the duration of the cardiac action potential and shorten the QT interval of the electrocardiogram. Block of the I_{Cl} by the Cl^- channel blocker anthracene-9-carboxylate increases the duration of the action potential in guinea pig ventricular myocytes during exposure to isoproterenol (Levesque *et al.*, 1993). By lengthening the cardiac action potential duration, future arylaminobenzoate derivatives might represent a new and unique group of class III antiarrhythmic agents that would be effective during sympathetic stimulation. The action of other putative CFTR channel blockers, such as sulfonylureas (Sheppard and Welsh, 1992; Venglarik *et al.*, 1996) and clofibrate acid analogues (Walsh and Wang, 1996), also will require careful attention.

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Send reprint requests to: Kenneth B. Walsh, Ph.D., Department of Pharmacology, University of South Carolina, School of Medicine, Columbia, SC 29208.