Voltage-dependent Block of the Cystic Fibrosis Transmembrane Conductance Regulator Cl^- Channel by Two Closely Related Arylaminobenzoates

N. A. McCarty, S. McDonough, B. N. Cohen, J. R. Riordan, N. Davidson, and H. A. Lester

From the Division of Biology 156-29, California Institute of Technology, Pasadena, California, 91125; The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8; and Department of Biochemistry and Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada M5G 1X8

ABSTRACT The gene defective in cystic fibrosis encodes a Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is blocked by diphenylamine-2-carboxylate (DPC) when applied extracellularly at millimolar concentrations. We studied the block of CFTR expressed in *Xenopus* oocytes by DPC or by a closely related molecule, flufenamic acid (FFA). Block of whole-cell CFTR currents by bath-applied DPC or by FFA, both at 200 μM, requires several minutes to reach full effect. Blockade is voltage dependent, suggesting open-channel block: currents at positive potentials are not affected but currents at negative potentials are reduced. The binding site for both drugs senses ~40% of the electric field across the membrane, measured from the inside. In single-channel recordings from excised patches without blockers, the conductance was 8.0 ± 0.4 pS in symmetric 150 mM Cl^-. A subconductance state, measuring ~60% of the main conductance, was often observed. Bursts to the full open state lasting up to tens of seconds were uninterrupted at depolarizing membrane voltages. At hyperpolarizing voltages, bursts were interrupted by brief closures. Either DPC or FFA (50 μM) applied to the cytoplasmic or extracellular face of the channel led to an increase in flicker at V_m = −100 mV and not at V_m = +100 mV, in agreement with whole-cell experiments. DPC induced a higher frequency of flickers from the cytoplasmic side than the extracellular side. FFA produced longer closures than DPC; the FFA closed time was roughly equal (~1.2 ms) at −100 mV with application from either side. In cell-attached patch recordings with DPC or FFA applied to the bath, there was flickery block at V_m = −100 mV, confirming that the drugs permeate through the membrane to reach the binding site. The data are consistent with the presence of a single binding site for both drugs, reached from either end of the channel. Open-channel block by DPC or FFA may offer tools for use with site-directed mutagenesis to describe the permeation pathway.

Address correspondence to Dr. Nael A. McCarty, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125.

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INTRODUCTION

The gene that is defective in cystic fibrosis encodes the cystic fibrosis transmembrane conductance regulator (CFTR); secondary structure predictions have suggested that the protein may serve as an ion channel (Riordan, Rommens, Kerem, Alon, Rozmahel, Grzelczak, Zielenski, Lok, Plavsic, Chou et al., 1989). Heterologous expression of the CFTR gene in epithelial and nonepithelial cells indicates that this gene encodes a Cl^- channel activated by the cAMP–protein kinase A pathway (Gregory, Cheng, Rich, Marshall, Paul, Hehir, Ostedgaard, Klinger, Welsh, and Smith, 1990; Rich, Anderson, Gregory, Cheng, Paul, Jefferson, McCann, Klinger, Smith, and Welsh, 1990; Anderson, Rich, Gregory, Smith, and Welsh, 1991*b*; Kartner, Hanrahan, Jensen, Naismith, Sun, Ackerley, Reyes, Tsui, Rommens, Bear, and Riordan, 1991). The characteristics of the channel (low conductance, linear current–voltage [*I-V*] relation, and selectivity for Cl^- over I^-) match those of the Cl^- permeability pathway now believed to be defective in cystic fibrosis (Welsh, 1990; Cliff, Schoumacher, and Frizzell, 1992).

This study concerns the permeation properties of the CFTR channel, as probed by small blocking molecules. By investigating the interaction of an open channel blocker with the pore of the protein, we hope to improve our understanding of CFTR function.

Several classes of molecules have been developed as inhibitors of various kinds of Cl^- channels (for review, see Greger, 1990). The disulfonic stilbenes, first developed as inhibitors of the red blood cell anion exchanger, block the outward rectifier Cl^- channel of epithelial cells (Singh, Afink, Venglarik, Wang, and Bridges, 1991; Tilmann, Kunzelmann, Fröbe, Cabantchik, Lang, Englert, and Greger, 1991). The phenoxyacetic acid derivative, indanyloxyacetic acid 94, was developed as a probe for isolation of an epithelial Cl^- channel (Landry, Reitman, Cragoe, and Al-Awqati, 1987). Anthracene-9-carboxylate is a useful inhibitor of the voltage-gated Cl^- channel of skeletal muscle, but is ineffective on the Cl^- channels of canine trachea and shark rectal gland, two chloride-secreting tissues (see Cabantchik and Greger, 1992). The arylaminobenzoates were developed by Greger and co-workers, who showed that diphenylamine-2-carboxylate (DPC) inhibited the basolateral Cl^- channel of renal thick ascending limb cells (DiStefano, Wittner, Schlatter, Lang, Englert, and Greger, 1985). DPC and its congeners are effective inhibitors of several epithelial Cl^- channels (Wangemann, Wittner, DiStefano, Englert, Lang, Schlatter, and Greger, 1986; Bijman, Englert, Lang, Greger, and Frömter, 1987; Tilmann et al., 1991).

The disulfonic stilbene, DIDS, is not an effective blocker of CFTR when applied to the bath (Kartner et al., 1991; Cunningham, Worrell, Benos, and Frizzell, 1992). In contrast, DPC has been used in several CFTR heterologous expression studies in which it inhibited whole-cell currents at fairly high concentrations (Rich et al., 1990). However, the single-channel and molecular bases of blockade of CFTR have not yet been described. In this report, we describe results from our studies of CFTR using two closely related arylaminobenzoates (DPC and flufenamic acid [FFA]; Fig. 1) as probes to investigate permeation properties of the channel. We find that both DPC and FFA block the CFTR channel in a voltage-dependent manner by introducing brief closures in the open-channel bursts. Analyses of the voltage dependence and

kinetics of drug–channel interactions allow us to suggest possible mechanisms for blockade of the pore by DPC and FFA. These results will serve as a basis for future studies involving site-directed mutagenesis of proposed pore-lining segments of the CFTR protein.

Portions of these data have been published previously in abstract form (McCarty, Cohen, Quick, Riordan, Davidson, and Lester, 1992; McDonough, McCarty, Riordan, Davidson, and Lester, 1993).

MATERIALS AND METHODS

Preparation of Oocytes and cRNA

Stage V–VI oocytes from female *Xenopus* were prepared as described (Quick, Naeve, Davidson, and Lester, 1992) and incubated at 18°C in incubation medium (see below). For the first day after isolation, incubation medium also contained 5% horse serum (Quick et al., 1992). In some cases, horse serum was continued for several days. In all cases, however, serum was removed at least 8 h before recording. cRNA was prepared from a construct carrying the full coding region of CFTR in a pSP64(poly A) vector (Bear, Duguay, Naismith, Kartner, Hanrahan, and Riordan, 1991). Oocytes were injected with 1–2 ng or 12–25 ng cRNA for whole-cell or single-channel recordings, respectively, in a volume of ~50 nl. Recordings were made 48–96 h after injection.

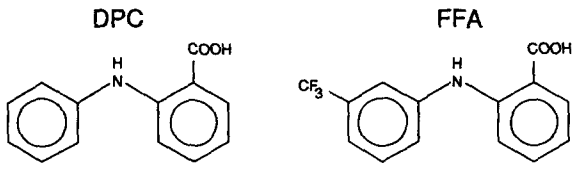

FIGURE 1. Molecular structures of the two blockers used in this study: DPC and FFA. FFA differs from DPC only with the addition of a trifluoromethyl group *meta* to the amine.

In experiments using heterologous expression of the human beta-2 adrenergic receptor (β_2-AR), 0.1–5 ng of this cRNA was injected along with CFTR. Injection of higher concentrations of β_2-AR cRNA in CFTR-injected oocytes led to spontaneous oscillations of holding current. This was probably due to spontaneous activation of the beta receptor, leading to high concentrations of cAMP, and could be prevented by incubation in propranolol (50 μM) for 8 h before recording.

Electrophysiology

Standard two-electrode voltage clamp techniques were used to study whole-cell currents. Electrodes were pulled in four stages from borosilicate glass (Sutter Instrument Co., Novato, CA) and filled with 3 M KCl. Pipette resistances measured 0.5–2.5 MΩ in bath solution. Membrane voltages were typically held at either −30 or −45 mV and stepped to a series of test potentials by an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA). Currents filtered at 500 Hz were stored on videotape after digitization or were acquired directly on-line using the Clampex program of pCLAMP (Axon Instruments, Inc.).

DPC and FFA also block the endogenous Ca^{2+}-activated Cl^- conductance, but with time course and voltage dependence different from CFTR blockade. Blockade of the endogenous conductance was complete within 1–2 min of exposure, suggesting an action from the outer surface of the membrane. The effects of DPC and FFA were not dependent on voltage: inward and outward currents were inhibited equally (data not shown). These results are consistent with

the initial description of blockade of the oocyte channel by FFA and niflumic acid (White and Aylwin, 1990). It should be noted, however, that Wu and Hamill (1992) recently reported a slight voltage dependence of the blockade of the endogenous channel in oocytes by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a blocker related to DPC and FFA. In our experiments, cAMP-activated currents were at least five times greater than the background currents; therefore, blockade of the endogenous channel produced negligible distortion in the overall effect of DPC and FFA.

For single-channel recordings, oocytes were shaken in stripping solution (see below) for 15 min. The vitelline membrane was then removed manually using fine forceps. Recordings were performed in both cell-attached and excised inside-out modes using electrodes pulled in four stages from borosilicate glass (Sutter Instrument Co.). Pipette resistances ranged from 10 to 40 MΩ. Seal resistances ranged from 20 to ~200 GΩ. Patch currents were measured with an Axopatch 1D amplifier (Axon Instruments, Inc.). Data were filtered at 2–2.5 kHz and stored on videotape. For subsequent analysis, records were filtered at 1 kHz with an 8-pole Bessel filter (model 902; Frequency Devices Inc., Haverhill, MA) and digitized at 100 μs per point during acquisition by the Fetchex program of pClamp (Axon Instruments, Inc.). In the single-channel traces shown, inward currents (anions passing from the cytoplasm to the external solution) are shown as negative (downward) deflections from baseline. Voltages in single-channel experiments are indicated as transmembrane voltage (internal minus external). In cell-attached recordings, we assume that the resting cell membrane potential is zero.

Analysis

Whole-cell current records were analyzed using the Clampfit program of pClamp (Axon Instruments, Inc.). For construction of *I-V* curves and calculation of voltage dependence of block, data were taken from the latter half of a 75-ms step to each potential and averaged. The current at each potential before cAMP stimulation and after washout was subtracted to determine cAMP-dependent current. For single-channel measurements, digitized Fetchex records were analyzed to produce idealized records and histogram files by the IPROC program (Axon Instruments, Inc.) (Sachs, Neil, and Barkakati, 1982; Sachs, 1983). IPROC uses a cubic splining function to interpolate between data points. Transition analysis used an open/closed border midway between the open and closed levels. The histogram files generated by IPROC were analyzed by NFITS (Island Products, Galveston, TX), a nonlinear least-squares curve-fitting procedure.

For calculation of open probability within a burst (P_o), closings >5 ms defined the end of a burst, since longer bursts often exceeded the IPROC burst memory. Bursts with baseline shift or more than one channel were not used for P_o calculations. System dead time was calculated and experimentally confirmed to be ~0.3 ms for a corner frequency (F_c) = 1 kHz. In closed-time histograms, curve-fitting began with the first bin past the dead time, the bin from 0.3 to 0.4 ms. Time constants were then taken from the extrapolated curves. Unless noted otherwise, values given are means ± SEM.

Solutions

The bath solution for whole-cell studies was ND96 without added Ca^{2+} salts, containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES, pH 7.5. Incubation medium was ND96 with added $CaCl_2$ (1.5 mM), Na-pyruvate (2.5 mM), and gentamycin (50 μg/ml), pH 7.5. The hypertonic stripping solution contained (mM): 200 monopotassium aspartate, 20 KCl, 1 $MgCl_2$, 10 EGTA, and 10 HEPES-KOH, pH 7.2. For patch clamp experiments, the pipette contained (mM): 150 *N*-methyl-D-glucamine·Cl, 0.5 $MgCl_2$, and 10 TES, pH adjusted to 7.4 with Tris. The intracellular solution for inside-out patches contained (mM): 150 NMDG·Cl, 1.1 $MgCl_2$, 2

Tris·EGTA, 1 MgATP (from equine muscle, < 1 ppm vanadium), and 10 TES, pH 7.4, and in some cases, 2 MgATP and 10 NaF. Bath solutions were delivered by gravity feed at a rate of ~3 ml/min. Intracellular cAMP levels were increased by application of either 1 μM isoproterenol or the cAMP-elevating cocktail of (μM): 1–10 forskolin, 10–200 dibutyryl-cAMP, and 10–200 3-isobutyl-1-methylxanthine. Cytoplasmic (4.3 mM) and extracellular (7.3 mM) ethanol, cytoplasmic 10 mM NaF, and raising cytoplasmic ATP concentration from 1 to 2 mM had no effect on the channel kinetics measured; these data were combined to form the unblocked channel data. 4 mM ethanol was also without effect on whole-cell CFTR currents.

When patches containing activated CFTR channels were excised into ATP solution, channel activity frequently ran down over the course of seconds. We considered it likely that this was due to a membrane-bound phosphatase that reversed the phosphorylation of CFTR by protein kinase A, or perhaps to intrinsic phosphatase activity of CFTR. We found that addition of 10 mM NaF to the intracellular solution slowed, although did not prevent, this run-down.

DPC and FFA were dissolved in 100% ethanol at stock concentrations of 1.25 and 0.15 M, respectively.

Source of Reagents

Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO). TES and DPC (*N*-phenylanthranilic acid) were from Aldrich Chemical Co. (Milwaukee, WI); FFA was from Sigma Chemical Co. Tris was from Boehringer Mannheim Corp. (Indianapolis, IN) and serum was from Irvine Scientific (Santa Ana, CA).

RESULTS

Whole-Cell Recordings

Cyclic AMP-dependent Cl^- currents were strongly activated in oocytes injected with CFTR and stimulated by either (*a*) application of cAMP-stimulating cocktail in cells injected with CFTR alone or (*b*) exposure to isoproterenol in cells coinjected with the human β_2-AR and CFTR. In cells expressing β_2-AR alone or CFTR alone, exposure to 10 μM isoproterenol did not lead to activation of currents significantly greater than prestimulation control (data not shown). Similarly, exposure of noninjected cells to the cAMP cocktail was without effect.

We took steps to avoid significant contamination of our whole-cell records by the endogenous Ca^{2+}-activated Cl^- ($Cl_{(Ca)}$) channels (see Materials and Methods). We used nominally Ca-free solutions and recorded only from oocytes whose resting conductances were low (holding current at $V_m = -45$ mV ≤ 250 nA). CFTR currents were easily distinguished from currents through the endogenous $Cl_{(Ca)}$ channel, because (*a*) the latter typically showed some decay of outward current through time while CFTR currents were consistently time independent; and (*b*) the endogenous currents show pronounced outward rectification, but CFTR currents are characterized by a nearly linear *I-V* relation. The slightly sublinear conductance of CFTR currents (see below) observed in our experiments at strongly hyperpolarizing voltages probably arises from the lower value of cytoplasmic Cl^- concentration relative to that in the bath. Parenthetically, we found that prolonged clamping of oocytes at holding potentials more negative than −30 mV led to further decreases of conductance at highly negative potentials. Presumably this effect arose from reequilibration of intracellular [Cl^-] at the new holding potential.

Effects of DPC on whole-cell currents. DPC blockade of CFTR was slow and voltage dependent. Exposure of cAMP-activated oocytes to DPC led to reduction in inward holding current in two phases. The initial, fast phase was probably due to partial blockade of the $Cl_{(Ca)}$ channel in addition to partial block of CFTR from the outside. The magnitude of this phase varied depending on the size of the background $Cl_{(Ca)}$ current. The subsequent phase required 7–10 min and involved a further decline in

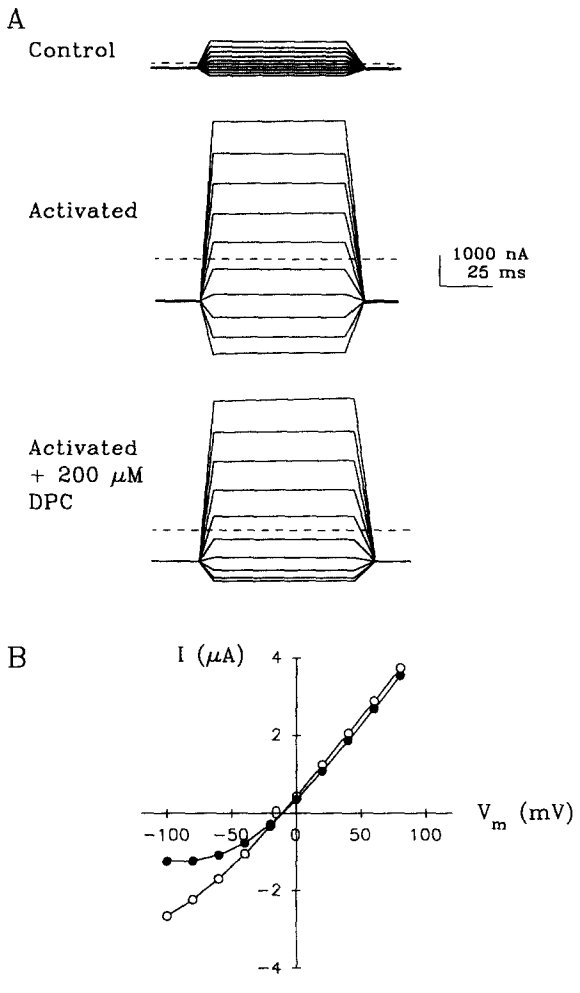

FIGURE 2. Blockade of whole-cell CFTR currents by 200 μM DPC. (*A*) Membrane currents elicited by stepping from the holding potential of −45 mV to a series of test potentials from −100 to +80 mV at 20-mV increments. Potential was held at the new value for 75 ms before returning to the holding potential. Capacitive currents have been subtracted. The dashed line in each trace indicates the zero current level. (*Top*) Control currents measured before cAMP activation. (*Middle*) CFTR currents measured after application of cAMP-stimulating cocktail. (*Bottom*) Activated CFTR currents measured 8 min after exposure to 200 μM DPC. All traces are from the same cell. Middle and bottom traces are not background-subtracted. (*B*) *I-V* relation for cAMP-dependent CFTR currents (*open circles*) and blocked currents (*filled circles*) after subtraction of control currents. Data points are from the average currents measured during the latter half of the voltage steps in *A*. Note the blockade of inward currents induced by DPC.

holding current. The extent of blockade of cAMP-stimulated holding current (at $V_m = -30$ mV) after this period was 37 ± 4% ($n = 6$) for 200 μM DPC and 80 ± 5% ($n = 8$) for 1 mM DPC. At equilibrium, DPC blockade of CFTR was clearly voltage dependent: there was pronounced outward rectification in the *I-V* relation (Fig. 2). Inward currents were greatly reduced, while outward currents were mostly unaffected. DPC and some congeners block nonselective cation channels (Gögelein, Dahlem,

Englert, and Lang, 1990) and inhibit prostaglandin biosynthesis (Flower, 1974), thus affecting intracellular metabolism. These secondary actions do not affect CFTR and are not apparent in the oocyte system at the drug concentrations used, because outward CFTR currents do not change over time. The persistence of outward current levels also served as an internal control to ensure that the activation level of CFTR did not decrease over the 7–10 min required for equilibrium blockade. The effects of DPC were fully reversible with a 10-min wash (data not shown).

The voltage dependence of block was calculated using average current values from the latter half of the 75-ms voltage steps to potentials ranging from −100 to +80 mV. Thus, the effect of voltage on the apparent dissociation constant was calculated as follows:

$$K_D(V) = [\text{drug}] \cdot \frac{I}{I_o - I}$$

where I_o is the current level at voltage V in cAMP-activated oocytes in the absence of blocker and I is the current level after several minutes of treatment with drug (Fig. 3). The calculated K_D at 0 mV averaged 912 ± 60 μM and K_D at −100 mV averaged 237 ± 34 μM for cells acutely exposed to 200 μM DPC (n = 7). For experiments with 1 mM DPC (for instance, Fig. 3), the data gave the following values: K_D(0 mV) = 1,116 ± 51 μM and K_D(−100 mV) = 346 ± 41 μM (n = 3). The electrical distance sensed by the blocker molecule, θ, is proportional to the slope of the curve of K_D versus voltage. Assuming valence = −1 and a single binding site for DPC, we calculated an average θ = 41 ± 7.0% as measured from the inside.

Effects of FFA on whole-cell currents. Oocytes expressing CFTR currents displayed a biphasic response to bath-applied 200 μM FFA. There was an initial fast block followed by a sizable increase in inward current after ~15 s, contrary to the expected blockade (n = 4, data not shown). We circumvented this secondary effect by injecting oocytes with 500 pmol EGTA (50 nl of a 10 mM stock, pH 8.4) 4–8 h before recording. Assuming a volume of 1 μl per oocyte, the final EGTA concentration was 0.5 mM. FFA applied to EGTA-injected oocytes did not cause increased current. With EGTA-injected oocytes, block of CFTR currents was similar to DPC block: there was an initial decline over 1–2 min, followed by a slower decline requiring several minutes to reach completion.

Release of calcium from intracellular stores, as shown to occur in some cell types (McDougall, Markham, Cameron, and Sweetman, 1988; Poronnik, Ward, and Cook, 1992) upon exposure to FFA and some congeners, but not DPC, could have caused the secondary increase in conductance via activation of the $Cl_{(Ca)}$ current. However, the effect of FFA on noninjected oocytes, or on CFTR-injected but unstimulated oocytes, was a simple fast block of the endogenous current (n = 8). We believe that the EGTA injection result indicates that the secondary effect of FFA might be due to potentiation of CFTR activation, either by (*a*) an additional intracellular signaling pathway or (*b*) modification of membrane recycling at the oocyte surface.

Like DPC, FFA blocked inward currents without large effects on outward currents (Fig. 4). Block of CFTR holding current (at −30 mV) by 200 μM FFA averaged 38 ± 7% (n = 6), as compared with 37 ± 4% for 200 μM DPC. The voltage dependence of blockade was also similar to that of DPC. Calculated K_D (0 mV) was 1,222 ± 88 μM

and K_D (−100 mV) was 289 ± 34 μM (n = 6). Voltage dependence of FFA block showed that θ = 41 ± 3%, similar to the value for DPC.

Single-Channel Recordings

Given the long time course of block and the pronounced voltage dependence for DPC and FFA, we considered it likely that these drugs block the CFTR channel by an interaction with the pore from the cytoplasmic side of the channel. This notion was

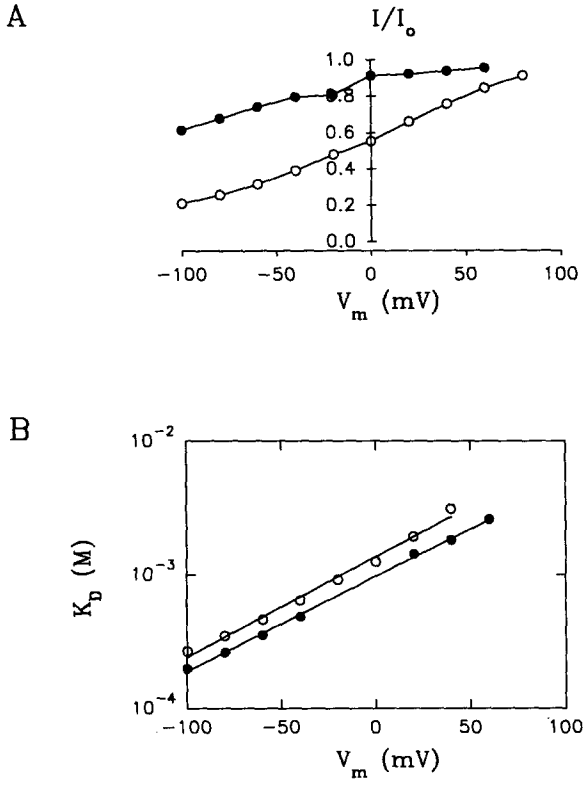

FIGURE 3. Voltage dependence of blockade by 200 μM DPC (*filled circles*) or by 1 mM DPC on a different cell (*open circles*). (*A*) Extent of blockade as a function of voltage. I is the current measured during exposure to drug, and I_o is the current measured in the absence of drug. (*B*) Effect of membrane voltage on the affinity of DPC for the channel. K_D was calculated according to the text. The line shown is the regression line. Some data points at voltages near the reversal potential (0 and −20 mV) were omitted from the analysis.

further investigated in patch clamp experiments where drug concentrations and site of delivery could be more closely controlled.

Single-channel CFTR currents were measured in oocytes activated by cocktail, heterologous expression of β_2-AR, or both (Fig. 5). CFTR channels are characterized by openings lasting up to several tens of seconds, a hallmark that renders this channel easily distinguished from contaminating channels (Bear et al., 1991). In symmetrical 150 mM Cl^-, the single-channel conductance was linear for voltages ±100 mV and ranged from 6.4 to 9.3 pS, averaging 8.0 ± 0.4 pS (n = 6). This channel was never seen in noninjected or mock-injected oocytes (n = 33). A subconductance state of ~60% the size of the main conductance was frequently observed at both V_m = −100 and V_m = +100 mV (see, for instance, asterisk in Fig. 7 *E*). The subconductance state often lasted for several seconds, though the time spent in the

subconductance state was less than ~5% of total time. At positive V_m, openings to the full conductance level were relatively uninterrupted. At negative V_m, bursts were highly interrupted by brief closures on a millisecond time scale. As shown in Fig. 5, the brief closures in unblocked channels are more prevalent in the cell-attached mode, perhaps reflecting blockade by a cytoplasmic molecule that is lost upon excision (see Tabcharani, Chang, Riordan, and Hanrahan, 1991). In the cell-attached mode, currents reversed close to the resting potential (data not shown). The currents shown in Fig. 5 at $V_m = \pm 100$ mV in the cell-attached mode, therefore, show slight

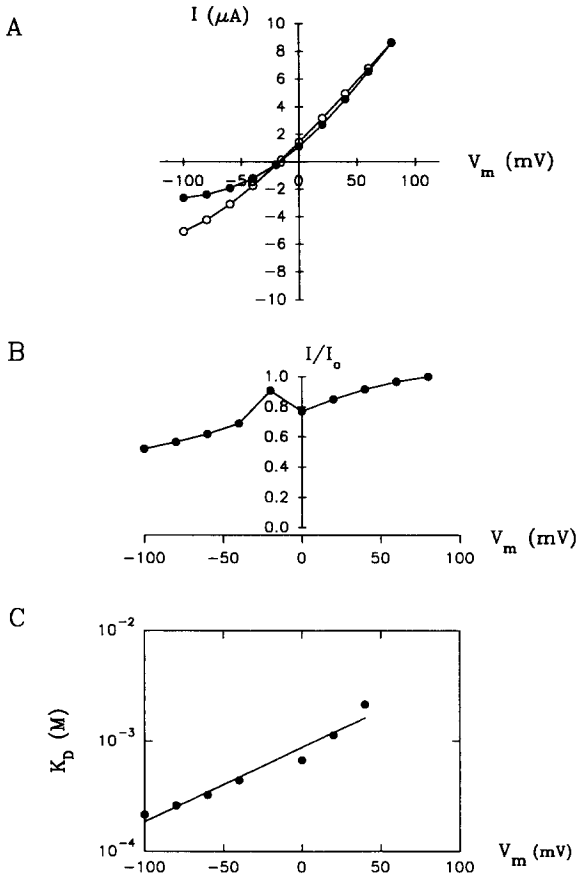

FIGURE 4. Blockade of CFTR currents by bath application of 200 μM FFA. (*A*) *I-V* relations determined as in Fig. 2. Shown are cAMP-dependent CFTR currents (*open circles*) and FFA-blocked currents (*filled circles*). (*B*, *C*) I/I_o and K_D as functions of voltage, respectively.

outward rectification, as expected from the fact that internal Cl^- concentration is several times lower than the pipette concentration of 150 mM.

Effect of cytoplasmic DPC and FFA on single-channel currents. As expected from the whole-cell data, 200 μM DPC applied directly to the cytoplasmic side of an excised patch led to reversible blockade of the single-channel openings at $V_m = -100$ mV, causing an increase in flicker (Fig. 6; see especially the expanded traces at right). Detailed analysis of block was carried out with 50 μM DPC or 50 μM FFA; these concentrations produced more clearly resolved open and closed intervals (Fig. 7,

Table I). DPC and FFA blockade showed the voltage dependence expected from whole-cell data described earlier; currents recorded at positive V_m were normal, but those at negative V_m were clearly blocked (Fig. 7).

Fig. 8 displays open- and closed-time histograms for control solutions and for cytoplasmic application of 50 μM DPC or FFA to patches containing activated CFTR channels in representative experiments; Table I summarizes these effects for several patches. The major point of these single-channel analyses is that blockade by either DPC or FFA is revealed as a decrease in mean open times and an increase in brief closed times. Despite the similar effects of DPC and FFA on whole-cell currents, the

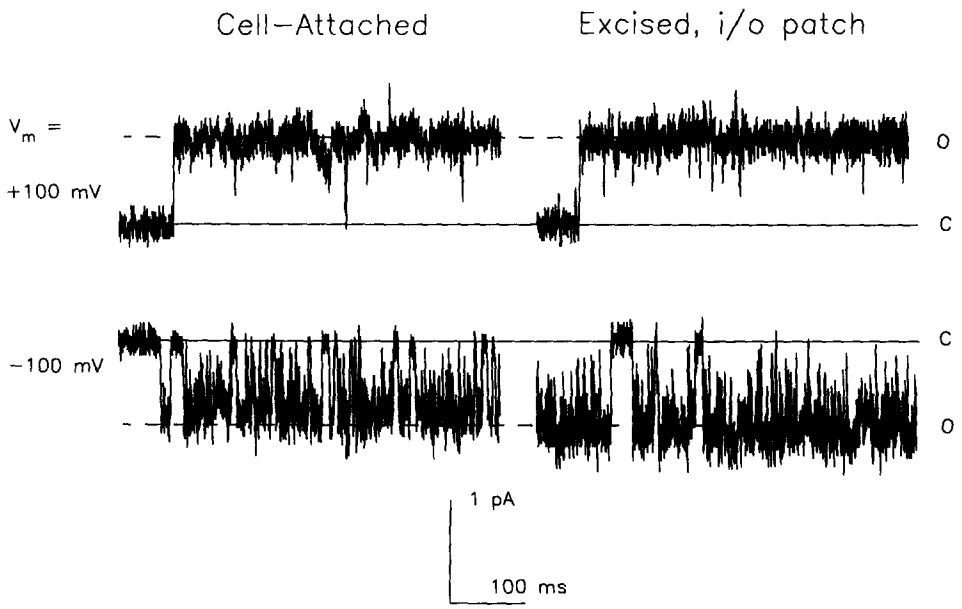

FIGURE 5. Comparison of single-channel currents measured in cell-attached and excised, inside-out patches at hyperpolarizing or depolarizing voltages. (*Left*) Currents measured in cell-attached configuration, with membrane potential (V_m) equal to +100 mV (*top*) and −100 mV (*bottom*). (*Right*) Currents measured in excised, inside-out patches, with V_m = +100 mV (*top*) and −100 mV (*bottom*). Traces are all from the same patch. Each trace shows 0.6 s of recording. The solid line indicates the closed level and the dashed line indicates the open level measured in excised mode.

details of the kinetic action of these two drugs differed. There was a more pronounced increase in flicker with FFA as compared with DPC, resulting in a clear additional closed-time constant. Hence, the mean closed time within a burst (τ_c) for CFTR channels was increased much more by FFA than by DPC. The average closed time for unblocked channels was 0.27 ms. For cytoplasmic DPC, the closed times were increased, but were still fit with a single exponential (τ_c). There was no reliable improvement in histogram fits with two exponential terms, presumably because the DPC-induced closures were several-fold more frequent than the endogenous closings

and yet differed from them by less than threefold in average duration. In contrast, closed times with cytoplasmic FFA were much longer, resulting in a fit with two exponentials: one corresponding to unblocked channels (τ_{c1}) and a longer constant from FFA block (τ_{c2}). The mean open time within a burst was greatly reduced by cytoplasmic DPC and FFA. However, mean open times showed no striking differences between drugs (Table I). Therefore, DPC and FFA produced roughly equal frequency of flickers when applied from the cytoplasmic side.

Effect of extracellular DPC and FFA on single-channel currents. Because we interpreted the fast component of whole-cell block by DPC and FFA to be due to block from outside of the cell, we also studied single channels in excised, inside-out patches

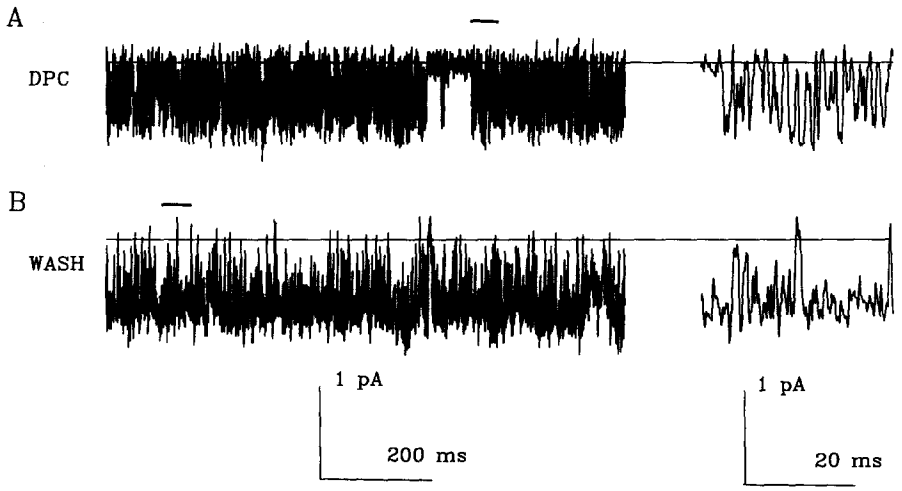

FIGURE 6. Reversibility of blockade by DPC applied to the cytoplasmic surface in excised, inside-out patches. The patch was excised directly into intracellular solution containing 200 μM DPC. Membrane potential (V_m) = −100 mV. (*A*) Currents measured in the presence of DPC in the cytoplasmic solution. (*B*) Washout of the DPC returned CFTR currents to the unblocked condition. Expanded traces at right are taken from the region indicated by the short bar above the unexpanded trace. The horizontal line indicates the closed level in both expanded and unexpanded traces.

with DPC or FFA in the pipette (i.e., bathing the external face of the channel). At 50 μM, neither drug reduced the open-channel conductance when applied from the outside. Both drugs caused a substantial increase in flicker at V_m = −100 mV (Fig. 9). Extracellular FFA blocked the channel more markedly than DPC (Fig. 9). The bursts with FFA in the pipette solution were more frequently interrupted by closures, some as long as several milliseconds. Closures of this duration were not apparent with DPC.

Dwell time analysis of extracellular DPC and FFA on single-channel currents is illustrated for representative patches in Fig. 10 and summarized in Table I. From the pipette, FFA clearly induced longer closed times than did DPC. As was the case for cytoplasmically applied drugs, closed-time distributions with DPC are fit with one

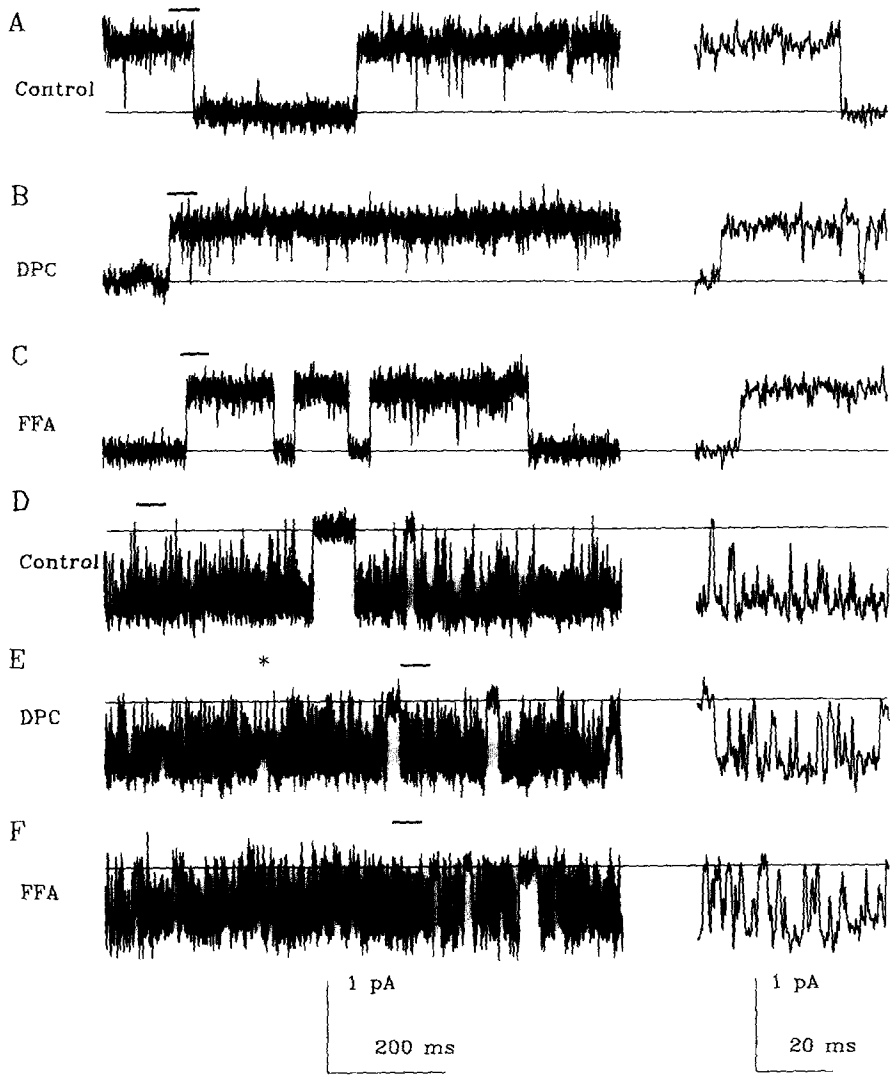

FIGURE 7. Effect of DPC and FFA at the cytoplasmic surface of excised, inside-out patches. (*A–C*) V_m = +100 mV. (*A*) No added blockers. (*B*) Cytoplasmic solution contained 50 μM DPC. (*C*) Cytoplasmic solution contained 50 μM FFA. (*D–F*) V_m = −100 mV. (*D*) Unblocked. (*E*) Cytoplasmic solution contained 50 μM DPC. (*F*) Cytoplasmic solution contained 50 μM FFA. Expanded traces at right are taken from the region indicated by the short bar above the unexpanded trace. Horizontal line indicates the closed level in both expanded and unexpanded traces. Asterisk in trace *E* denotes an example of a sojourn in the subconductance state.

TABLE I

Kinetic Effects of 50 μM DPC and FFA in Excised Patches at $V_m = -100$ mV

	Unblocked*	DPC		FFA	
		Cytoplasmic	Pipette	Cytoplasmic	Pipette
τ_o, *ms*	8.23 ± 1.4	2.17 ± 0.43	3.16 ± 0.56	2.42 ± 0.41	2.37 ± 0.10
τ_{c1}, *ms*	0.27 ± 0.01	0.62 ± 0.08	0.46 ± 0.05	0.28 ± 0.03	0.37 ± 0.08
τ_{c2}, *ms*				1.11 ± 0.05	1.41 ± 0.12
P_o‡	0.94 ± 0.01	0.69 ± 0.07	0.80 ± 0.03	0.75 ± 0.03	0.70 ± 0.02
n	6	4	3	4	3
Time analyzed, *s*	739	161	160	388	1,020

*Unblocked values combine data from all controls in the presence and absence of ethanol (7.3 mM in the pipette and 4.3 mM in the cytoplasm), 1 or 2 mM ATP, and 10 mM NaF, all of which had no effect.
‡Values for P_o are measured within a burst. The data presented exclude two records where it could not be determined: one each for FFA cytoplasmic and FFA pipette.

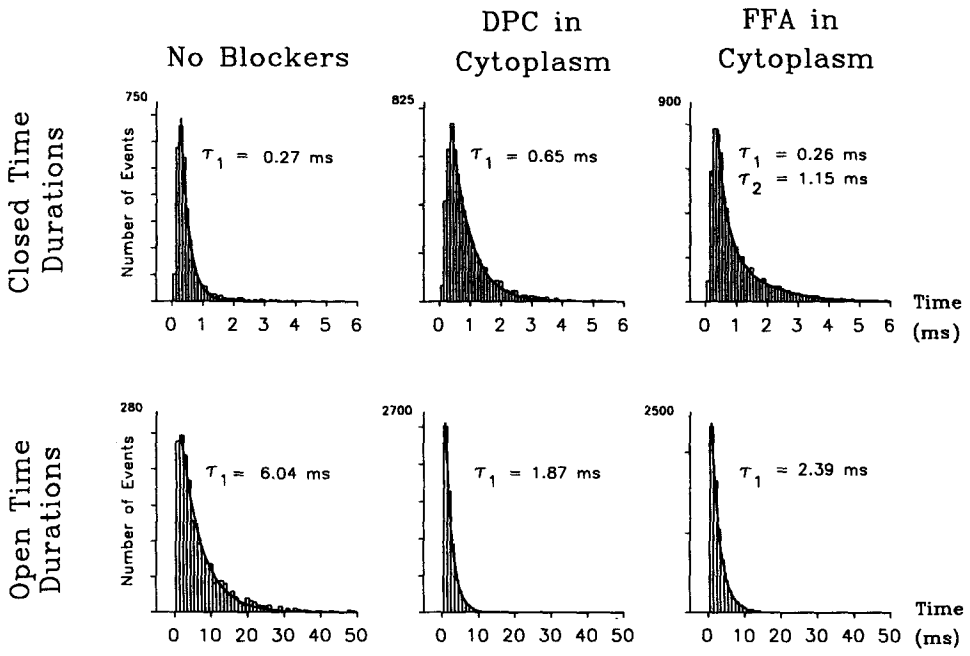

FIGURE 8. Summary of the effects of cytoplasmic drugs on CFTR kinetics in representative experiments. $V_m = -100$ mV. (*Top*) Histograms of closed-time durations within a burst. Value of τ shown is the average closed time calculated from fitting the histogram with one (τ_1) or two (τ_1 and τ_2) exponential components. On each histogram, the maximum range of the ordinate is labeled. (*Bottom*) Open-time duration histograms. Binwidth was 0.1 ms for closed-time histograms and 1 ms for open-time histograms. For cytoplasmically applied FFA, the ratio of areas under the curves for the short and long closed-time constants indicates that the latter accounted for 49 ± 2% of the closed events ($n = 4$).

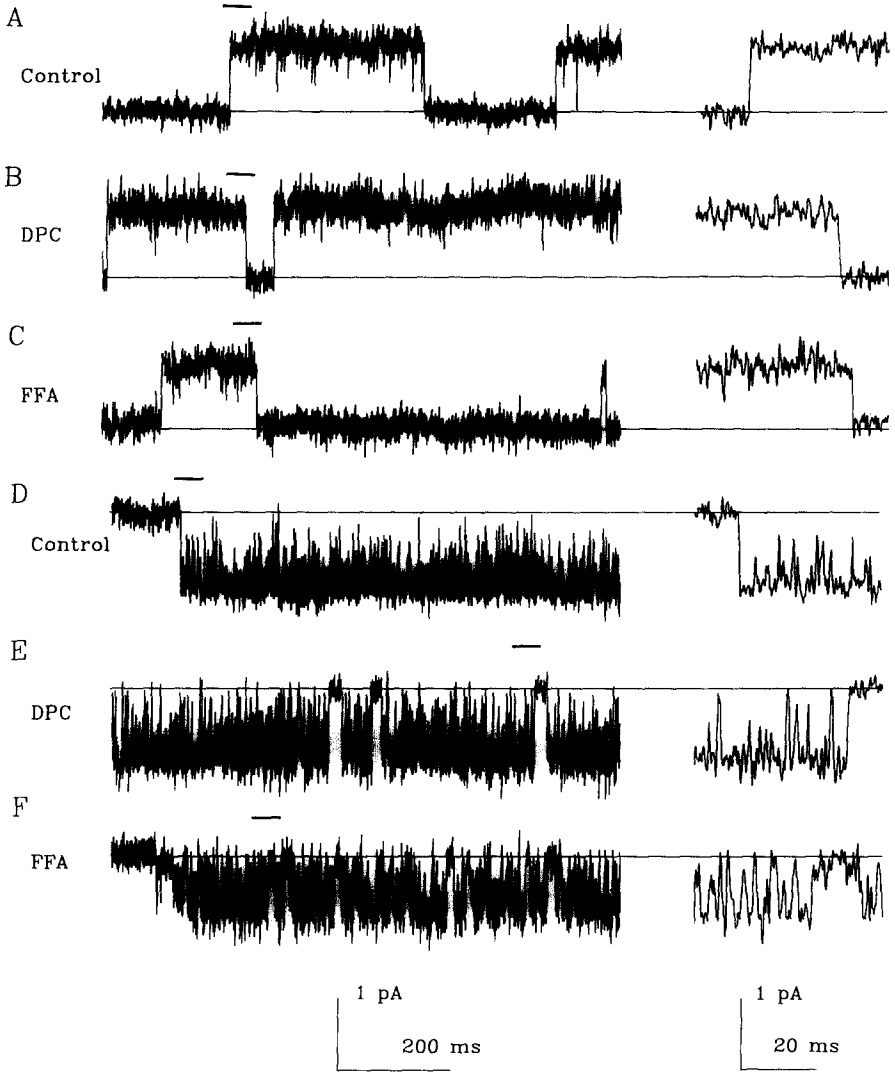

FIGURE 9. Effect of DPC and FFA at the external surface of excised, inside-out patches. (*A–C*) V_m = +100 mV. (*A*) No added blockers. (*B*) Pipette solution contained 50 μM DPC. (*C*) Pipette solution contained 50 μM FFA. (*D–F*) V_m = −100 mV. (*D*) No added blockers. (*E*) Pipette solution contained 50 μM DPC. (*F*) Pipette solution contained 50 μM FFA. Expanded traces at right are taken from the region indicated by the short bar above the unexpanded trace. Horizontal line indicates the closed level in both expanded and unexpanded traces.

exponential, while closed-time distributions with FFA are fit with two exponentials. The open-time distributions show that both DPC and FFA reduced the mean open time from 8.23 ms in the absence of blockers to 2.5 to 3 ms in the presence of blockers, with no significant difference between DPC and FFA.

Permeation of bath-applied DPC and FFA through the membrane. The slow phases of block by DPC and FFA in the whole-cell mode implied that both blockers were

membrane permeant. Cell-attached channels were more flickery than excised channels (Fig. 5), vitiating quantitative analysis of additional block by DPC or FFA. Nonetheless, to confirm that DPC can permeate through the plasma membrane to block CFTR from the inside, we activated channels in the cell-attached mode; the oocyte was then exposed to 200 μM DPC in the bath solution. After several minutes, channel openings were clearly interrupted at $V_m = -100$ (Fig. 11), but not at $V_m = +100$ mV (data not shown). FFA applied to the bath (50 μM) also blocked in the cell-attached mode (Fig. 11). Because the drug did not have access to the external side of the channels in the patch in this configuration, these results show that drug

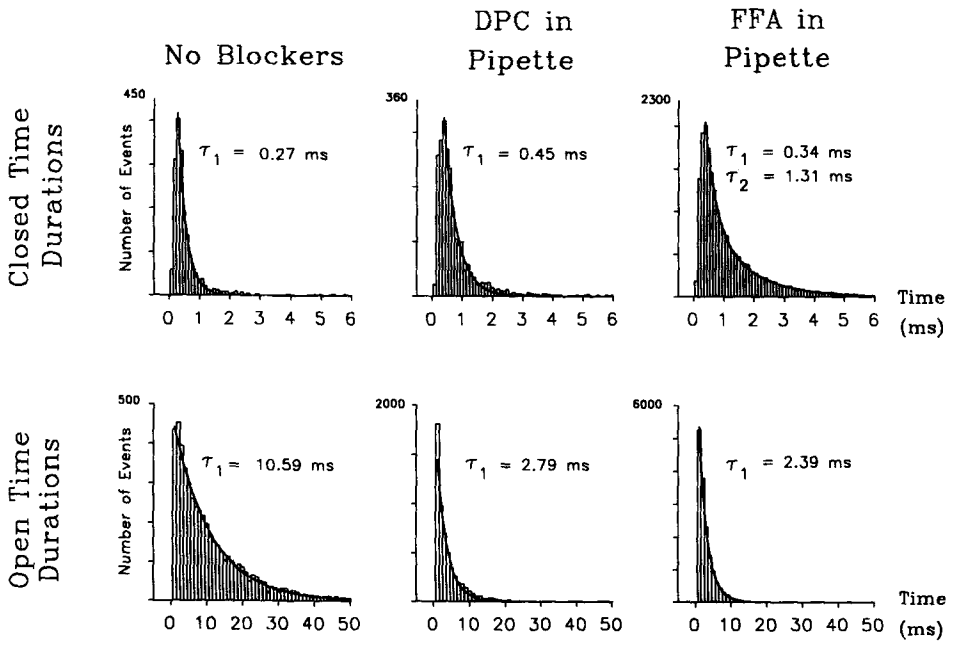

FIGURE 10. Summary of the effects of external drugs on CFTR kinetics in representative experiments. $V_m = -100$ mV. Axes and scales as in Fig. 8. (*Top*) Closed-time duration histograms. (*Bottom*) Open-time duration histograms. For externally applied FFA, the ratio of areas under the curves for the short and long closed-time constants indicates that the latter accounted for 45 ± 11% of the closed events ($n = 3$).

molecules can cross the membrane from the bath and access their binding site from the cytoplasmic side of the channel.

DISCUSSION

The gene that is defective in cystic fibrosis encodes a Cl^- channel that is regulated by the cAMP–protein kinase A pathway. That the protein itself is a Cl^- channel is indicated by the findings that: (*a*) individuals carrying mutant CFTR gene alleles often show decreased Cl^- transport in affected tissues (Quinton, 1983; Boat, Welsh, and Beaudet, 1989); (*b*) expression of the CFTR gene in cells from a variety of

nonepithelial and cystic fibrosis epithelial sources results in the appearance of a cAMP-activatable Cl^- conductance of specific character (Anderson et al., 1991*b*; Bear et al., 1991; Kartner et al., 1991; Cunningham et al., 1992); (*c*) site-directed mutations in the CFTR cDNA result in discrete changes in Cl^- channel function (e.g., Anderson, Gregory, Thompson, Souza, Paul, Mulligan, Smith, and Welsh, 1991*a*; Cheng, Rich, Marshall, Gregory, Welsh, and Smith, 1991; Rich, Gregory, Anderson, Manavalan, Smith, and Welsh, 1991); and (*d*) purified CFTR protein provides functional Cl^- channels upon incorporation into planar lipid bilayers (Bear, Li, Kartner, Bridges, Jensen, Ramjeesingh, and Riordan, 1992).

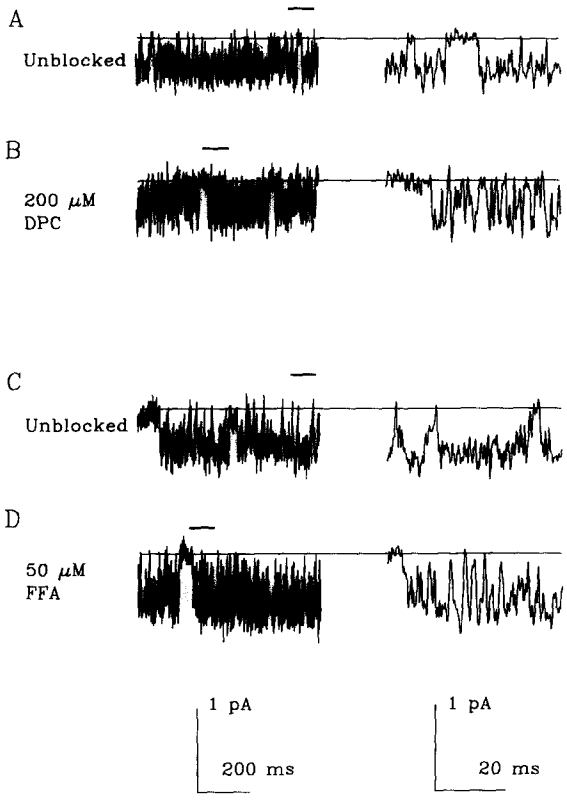

FIGURE 11. Blockade of CFTR currents by DPC and FFA in the cell-attached mode. Patch currents measured before (*A*) and after (*B*) application of 200 μM DPC to the bathing medium. Expanded traces at the right are taken from the area indicated by the short bar above the unexpanded trace. The horizontal line indicates the closed level for channels at both time scales. *C* and *D* show currents measured before and after, respectively, application of 50 μM FFA to the bath. *A* and *B* are from the same patch, as are *C* and *D*. In each case, $V_m = -100$ mV. Traces shown in *B* and *D* were obtained 1–2 min after application of the drugs.

This study characterizes the blocking action of two arylaminobenzoates, DPC and its derivative, FFA. Both DPC and FFA block CFTR in a voltage-dependent manner by mechanisms to be discussed below. Thus, DPC and FFA will be useful as probes to characterize the pore of the CFTR channel.

In pioneering studies on heterologous expression of the CFTR gene, 3 mM DPC caused blockade of Cl^- currents in cystic fibrosis airway epithelial cells, CHO cells, and HeLa cells transfected with CFTR (Rich et al., 1990; Anderson et al., 1991*b*). Blockade was not immediate, and voltage dependence was not investigated. NPPB also blocks the CFTR-like channel found in pancreatic duct cell apical membranes

(Gray, Pollard, Harris, Coleman, Greenwell, and Argent, 1990), T84 cell membranes (Tabcharani, Low, Elie, and Hanrahan, 1990), and *Xenopus* oocytes injected with mRNA from shark rectal gland (Sullivan, Swamy, and Field, 1991). In contrast, both DPC and NPPB were only marginally effective at blocking CFTR expressed in oocytes in one study (Cunningham et al., 1992). However, this contradictory finding may be due to the fact that Cunningham et al. (1992) determined cAMP-dependent currents by stepping the voltage to 80 mV depolarized from the resting potential. Hence, only outward currents were measured, and our results show that DPC is not an effective blocker of outward currents. We have found no reports on blockade of CFTR by FFA.

In contrast to block of CFTR by arylaminobenzoates, disulfonic stilbenes appear to be ineffective. CFTR currents are insensitive to block by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) upon expression in oocytes (Cunningham et al., 1992) and Sf9 cells (Kartner et al., 1991). The small-conductance CFTR-like channels in pancreatic duct cell apical membranes (Gray et al., 1990) and T84 cell membranes (Tabcharani et al., 1990) were also insensitive to block by DIDS. However, disulfonic stilbenes are effective blockers of the intermediate conductance outward rectifier anion channel of epithelia (Singh et al., 1991; Tilmann et al., 1991) and the Ca^{2+}-activated Cl^- channel in several cell types. In oocytes, 50 μM DIDS blocked the background conductance in a voltage-independent manner (Cunningham et al., 1992).

Voltage-dependent Block of Macroscopic Currents

Whole-cell currents in CFTR-injected oocytes activated by cAMP were inhibited by both DPC and FFA. Blockade by both drugs was strongly voltage dependent: the K_D decreased as transmembrane potential became more negative at a rate of e-fold per ~60 mV. This voltage dependence contrasts with the much weaker, voltage-independent blockade of the endogenous Ca^{2+}-activated Cl^- channels by DPC. CFTR block by DPC and FFA required several minutes to reach maximal effect. Blockade occurred in two phases: a fast phase due to partial block from the external side of the channel, followed by a slower phase, presumably due to block from the cytoplasmic side after drug permeation through the plasma membrane. The voltage dependence of blockade, as determined by the variation in K_D with voltage, indicates that DPC and FFA molecules sense a similar fraction (0.4–0.5) of the electrical field across the membrane and hence may share a common binding site.

Voltage-dependent Block of Single-Channel Currents

Single-channel data indicate that both DPC and FFA block CFTR by increasing the frequency of brief nonconducting periods: the current traces become more flickery without a reduction in the apparent single-channel conductance. Thus, DPC and FFA may be classified as "intermediate" drugs, in the terminology of Hille (1992), regarding the kinetics of drug–receptor interaction. Interestingly, the subconductance state showed much less flicker than the main state upon application of drugs to either side (data not shown). This effect has not been studied systematically.

As indicated in Table I, the block of DPC and FFA is revealed both by (*a*) an increase in average closed times and (*b*) a decrease in average open times in the presence of blockers. In Table I, the average closed times for all four drug configurations were significantly different from closed times for unblocked channels

($P < 0.05$, unpaired t test). The same is true for average open times, with the exception of open times with external DPC, the least potent blocking condition ($P = 0.051$). Single-channel measurements confirm the whole-cell data: the flickery block occurs only at negative membrane potentials. Block of single channels by DPC was reversible, as was the case for whole-cell measurements. Increasing order of potency of block at a given concentration and voltage is as follows: DPC applied externally, DPC internally, FFA internally, and FFA externally.

Open-Channel Block

Two general classes of block involve either (*a*) direct block of conduction due to binding within the pore itself or (*b*) allosteric inhibition due to binding outside the pore. That FFA and DPC blockade is voltage dependent, with an apparent binding site 40–50% of the electrical distance from the cytoplasmic face, suggests that the blockade actually occurs within the channel. In addition, the blocked single-channel traces resemble classic open-channel block; brief blocked times would represent the residence time of the blocker on its site. We have no formal proof of this mechanism, however; future experiments will test this suggestion by examining antagonism of the blockade by permeant ions.

Assuming for the moment that open-channel block is the mechanism, a simple kinetic model may be discussed. A drug molecule, T, binds to a single class of receptor sites, R, as follows:

$$T + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TR \qquad K_D = \frac{k_{-1}}{k_1}$$

where k_1 is the second-order bimolecular rate constant for binding, k_{-1} is the first-order unimolecular rate constant for dissociation, and K_D is the equilibrium dissociation constant for the drug–receptor complex. Because $[T]k_1 = 1/\tau_o$ and $k_{-1} = 1/\tau_c$, we can estimate the apparent dissociation constant from the single-channel data at a given drug concentration and at a given membrane voltage, $K_D(V)$ as

$$K_D(V) = \frac{1/\tau_c}{1/\tau_o}[T]$$

Applying values for τ_o and τ_{c1} or τ_{c2} at $V_m = -100$ mV from Table I, we calculate values for $K_D(-100)$ under the following conditions: for DPC_{ext}, 345 μM; for DPC_{cyto}, 175 μM; for FFA_{cyto}, 105 μM; and for FFA_{ext}, 85 μM, where the subscripts ext and cyto refer to drugs in the extracellular and cytoplasmic solutions, respectively. This quantitative order of potency agrees with the perceived order of potency listed above and with the similarity in values for K_D (−100 mV) measured from block of whole-cell currents, which averaged 237 ± 34 and 289 ± 33 μM for DPC and FFA, respectively. The single-channel data suggest that FFA binds two to three times more tightly than DPC; the whole-cell data suggest roughly equal equilibrium dissociation constants. We believe that the single-channel data give the more accurate picture because the drug concentrations are more directly controlled in excised-patch experiments.

The similarity in τ_o values for DPC and FFA in all four configurations suggests that $k_1 \approx 6 \times 10^7\ s^{-1}$, close to the value of $2 \times 10^7\ s^{-1}$ determined for open-channel

blockers of the acetylcholine receptor channel (Charnet, Labarca, Leonard, Vogelaar, Czyzyk, Gouin, Davidson, and Lester, 1990). The most striking difference in kinetic effects between DPC and FFA in the four configurations is seen in the closed-time constants. FFA apparently remains bound to its site several times longer than does DPC.

Access through the Conduction Pathway?

How do the blockers reach their binding sites? The most important constraint for modeling this process is that the blockers appear to reach their binding site from either side of the membrane; furthermore, kinetics of FFA, which yields equal voltage dependence and residence time with application from either side, suggest that the binding site is the same from either side. Possible routes of access are (*a*) through the conducting pathway, or (*b*) through the membrane itself. There are precedents for both routes (Hille, 1992).

Permeant blocker; asymmetric energy barriers. One possible view of block in the CFTR channel invokes permeant blockers and asymmetric energy barriers, similar to more detailed models that explain block of Na^+ channels by protons (Woodhull, 1973) and block of retinal cyclic nucleotide–gated cation channels by divalent cations (Zimmerman and Baylor, 1992). In its simplest form, this model would incorporate two barriers (with energy peaks E_{outer} and E_{inner}) flanking one well located at a distance ~0.4–0.5 from the inside edge. The well depth for DPC is shallower than for FFA, accounting for the longer residence time of FFA (longer closed-time constant, τ_c). At zero transmembrane potential, the external energy barrier E_{outer} is less than E_{inner}. At $V_m = -100$ mV, the inner and outer energy barriers experienced by a blocker molecule are of equal height, so that the drugs can reach the binding site equally well from either side, accounting for the similar forward binding rates, k_1. Regardless of the side of drug application, upon unbinding at negative V_m the (negatively charged) drug molecules are more likely to exit down the electrical gradient, over the external barrier. At positive membrane voltages, the internal barrier is lowered to the point where externally applied drug rapidly exits to the internal solution down the electrical gradient, resulting in little or no blockade. Internally applied drugs cannot overcome the larger internal barrier against the electrical gradient. The whole-cell data do show a small degree of block by both DPC and FFA at depolarizing voltages (see Figs. 3 and 4), which could represent very short-lived residence of the blocker on the binding site; this brief block would not be resolved in our single-channel measurements.

The asymmetric barrier model explains the counterintuitive result that externally applied DPC and FFA are each capable of entering the channel and reaching their binding site against the applied membrane field. Similarly, Ca^{2+} blocks the retinal cyclic nucleotide–gated channel although the apparent electrical gradient does not favor its entry into the channel (Zimmerman and Baylor, 1992).

Block due to lock-in. If the pore contains multiple anion binding sites, chloride occupancy of binding sites could impede dissociation of FFA or DPC from the pore. Such a lock-in phenomenon has been studied for the calcium-activated potassium channel of rat skeletal muscle, most recently by Neyton and Miller (1988*a*, *b*). However, CFTR channels are characterized by a low conductance and a low degree of

size selectivity, implying that there may not be multiple anion binding sites within the pore.

Regardless of the exact mechanism for blocker binding, these models suggest that DPC and FFA are permeant blockers: they can exit in the opposite direction from their entrance. Their action is thus more complex than that of quaternary ammonium compounds at nicotinic acetylcholine receptors or voltage-gated Na^+ channels.

Access through the Lipid Phase?

Possibly the drugs do not reach their binding sites through the conduction pathway from the external surface, but rather through the membrane phase. The membrane route might involve the uncharged form of the drugs. The charged form of the drugs would be the blocking form so that the observed voltage dependence would still be expected. This mechanism would be tested with measurements of pH dependence.

DPC vs. FFA

We consider it likely that the major interaction between the drug molecules and the walls of the pore involves the negative charge of the carboxylate moiety. However, kinetic differences between FFA and DPC indicate that substitution of a trifluoromethyl group influences binding energy, either through a direct interaction of the CF_3 group with the channel or through an altered charge distribution on FFA as compared with DPC.

FFA blocked whole-cell currents somewhat faster than DPC; similarly, externally applied FFA blocks single channels more effectively than externally applied DPC. The difference in K_D values between whole-cell and single-channel measurements is probably due to the presence of blockers on both sides of the membrane in the whole-cell experiments, counteracting the difference in sidedness observed in single-channel experiments. We do not know the exact intracellular concentration of drugs even after prolonged incubation, although block of CFTR in cell-attached patch mode by bath-applied DPC and FFA indicates that both drugs are capable of crossing the membrane.

The mechanism of voltage-dependent block by FFA and DPC needs to be addressed more fully in future experiments using a wider range of pH, permeant ion concentrations, and membrane potentials. It is also of interest to determine whether site-directed mutations of the CFTR gene can be identified that alter the blocking process. Voltage-dependent blockade of CFTR channels by FFA and DPC constitutes a promising tool for structure–function studies of CFTR.

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