Effect of ATP-sensitive K^+ Channel Regulators on Cystic Fibrosis Transmembrane Conductance Regulator Chloride Currents

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ABSTRACT The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel that is regulated by cAMP-dependent phosphorylation and by intracellular ATP. Intracellular ATP also regulates a class of K^+ channels that have a distinct pharmacology: they are inhibited by sulfonylureas and activated by a novel class of drugs called K^+ channel openers. In search of modulators of CFTR Cl^- channels, we examined the effect of sulfonylureas and K^+ channel openers on CFTR Cl^- currents in cells expressing recombinant CFTR. The sulfonylureas, tolbutamide and glibenclamide, inhibited whole-cell CFTR Cl^- currents at half-maximal concentrations of ~150 and 20 μM, respectively. Inhibition by both agents showed little voltage dependence and developed slowly; >90% inhibition occurred 3 min after adding 1 mM tolbutamide or 100 μM glibenclamide. The effect of tolbutamide was reversible, while that of glibenclamide was not. In contrast to their activating effect on K^+ channels, the K^+ channel openers, diazoxide, BRL 38227, and minoxidil sulfate inhibited CFTR Cl^- currents. Half-maximal inhibition was observed at ~250 μM diazoxide, 50 μM BRL 38227, and 40 μM minoxidil sulfate. The rank order of potency for inhibition of CFTR Cl^- currents was: glibenclamide > BRL 38227 ≅ minoxidil sulfate > tolbutamide > diazoxide. Site-directed mutations of CFTR in the first membrane-spanning domain and second nucleotide-binding domain did not affect glibenclamide inhibition of CFTR Cl^- currents. However, when part of the R domain was deleted, glibenclamide inhibition showed significant voltage dependence. These agents, especially glibenclamide, which was the most potent, may be of value in identifying CFTR Cl^- channels. They or related analogues might also prove to be of value in treating diseases such as diarrhea, which may involve increased activity of the CFTR Cl^- channel.

INTRODUCTION

Mutations in a single gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) cause the disease cystic fibrosis (CF) (Riordan, Rommens, Kerem,

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Alon, Rozmahel, Grzelczak, Zielenski, Lok, Plavsic, Chou et al., 1989). Amino acid sequence analysis and comparison with other proteins suggests that CFTR consists of five domains: two membrane-spanning domains, each composed of six transmembrane segments; an R domain, which contains several consensus sequences for phosphorylation by cAMP-dependent protein kinase (PKA); and two nucleotide-binding domains (NBDs), which are predicted to interact with ATP. Such analyses also suggest that CFTR belongs to a family of proteins that have been called the traffic ATPases (Ames, Mimura, and Shyamala, 1990) or the ABC transporters (Hyde, Emsley, Hartshorn, Mimmack, Gileadi, Pearce, Gallagher, Gill, Hubbard, and Higgins, 1990). Some members of this family hydrolyze ATP to transport substrate across cell membranes.

Recent work suggests that CFTR is a Cl^- channel. That conclusion is based on four observations. First, expression of recombinant CFTR in a wide variety of epithelial and nonepithelial cells generated cAMP-activated Cl^- channels (Drumm, Pope, Cliff, Rommens, Marvin, Tsui, Collins, Frizzell, and Wilson, 1990; Rich, Anderson, Gregory, Cheng, Paul, Jefferson, McCann, Klinger, Smith, and Welsh, 1990; Anderson, Rich, Gregory, Smith, and Welsh, 1991*c*; Bear, Duguay, Naismith, Kartner, Hanrahan, and Riordan, 1991; Kartner, Hanrahan, Jensen, Naismith, Sun, Ackerley, Reyes, Tsui, Rommens, Bear, and Riordan, 1991). Second, the properties of cAMP-regulated Cl^- channels generated by recombinant CFTR were the same as those of cAMP-regulated Cl^- channels in the apical membrane of secretory epithelia, where the CF defect is located (Anderson, Sheppard, Berger, and Welsh, 1992). Third, mutation of specific basic residues within the membrane-spanning domains to acidic residues altered the channel's anion selectivity (Anderson, Gregory, Thompson, Souza, Paul, Mulligan, Smith, and Welsh, 1991*b*). Fourth, when purified recombinant CFTR was incorporated into planar lipid bilayers it displayed regulated Cl^- channel activity (Bear, Li, Kartner, Bridges, Jensen, Ramjeesingh, and Riordan, 1992).

The CFTR Cl^- channel is regulated by cAMP: in experiments using the whole-cell and cell-attached configurations of the patch-clamp technique, cAMP agonists activated CFTR Cl^- currents (Drumm et al., 1990; Rich et al., 1990; Anderson et al., 1991*c*; Bear et al., 1991; Berger, Anderson, Gregory, Thompson, Howard, Maurer, Mulligan, Smith, and Welsh, 1991; Kartner et al., 1991; Tabcharani, Chang, Riordan, and Hanrahan, 1991). This regulation occurs via phosphorylation, since addition of the catalytic subunit of cAMP-dependent protein kinase to the cytosolic surface of excised inside-out membrane patches activated CFTR Cl^- channels (Berger et al., 1991; Tabcharani et al., 1991). The R domain is the site of PKA-dependent regulation because four serine residues in the R domain are phosphorylated in vivo when cellular levels of cAMP increase (Cheng, Rich, Marshall, Gregory, Welsh, and Smith, 1991). Mutation of those serines to alanines abolishes cAMP-dependent activation (Cheng et al., 1991). Moreover, expression of CFTR in which part of the R domain has been deleted produces Cl^- channels that are constitutively active; that is, channel activation does not require an increase in cellular cAMP levels (Rich, Gregory, Anderson, Manavalan, Smith, and Welsh, 1991).

Nucleoside triphosphates, such as ATP, also regulate the CFTR Cl^- channel: once phosphorylated by PKA, micromolar concentrations of ATP are required to maintain

channel activity (Anderson, Berger, Rich, Gregory, Smith, and Welsh, 1991*a*). ATP activates the channel via a mechanism independent of both PKA and the R domain; regulation at the NBDs is an attractive explanation for this effect.

Intracellular ATP also regulates a class of K^+ channels (ATP-sensitive K^+ channels; K-ATP channels) (Noma, 1983; Cook and Hales, 1984; Ashcroft and Ashcroft, 1990). The opening of K-ATP channels in pancreatic β cells, myocytes, and some neurons is coupled to the cytoplasmic concentration of ATP. In contrast to the CFTR Cl^- channel where ATP activates the channel, micromolar concentrations of ATP inhibit K-ATP channels (Ashcroft and Ashcroft, 1990). Although the primary structure of K-ATP channels is presently unknown, their regulation by ATP suggests that they may share some similarity with CFTR Cl^- channels.

K-ATP channels have a distinct pharmacology: they are inhibited by sulfonylureas, such as tolbutamide and glibenclamide, a group of hypoglycemia-inducing drugs used to treat diabetes mellitus (Sturgess, Ashford, Cook, and Hales, 1985). K-ATP channels are also activated by a novel class of drugs known as K^+ channel openers (Edwards and Weston, 1990; Standen, Quayle, Davies, Brayden, Huang, and Nelson, 1989). These agents include cromakalim, a potent smooth muscle relaxant with hypotensive and bronchodilatory activity in vivo, as well as diazoxide, an antihypertensive drug also used to treat some pancreatic carcinomas (Dunne and Petersen, 1991).

In contrast, the pharmacology of CFTR Cl^- channels is less well defined. The search for modulators of CFTR Cl^- channels is important in at least two respects. First, no high affinity inhibitors of CFTR Cl^- channels have yet been identified; such inhibitors might be useful as agents for distinguishing CFTR Cl^- channels and as probes of the mechanism of ion permeation. Second, novel pharmacological activators of CFTR Cl^- channels might provide an important therapy for the defective Cl^- secretion across CF epithelia.

Thus, the goal of this study was to examine the effect of agents that might inhibit or activate CFTR Cl^- channels. Because both CFTR and K-ATP channels are regulated by ATP, we examined the effect of sulfonylureas and K^+ channel openers on CFTR Cl^- currents measured using the whole-cell, patch-clamp technique.

MATERIALS AND METHODS

For this study we used NIH 3T3 fibroblasts that had been stably infected with a retrovirus expressing either wild-type human CFTR (Gregory, Cheng, Rich, Marshall, Paul, Hehir, Ostedgaard, Klinger, Welsh, and Smith, 1990) or a CFTR mutant in NBD2 (CFTR-K1250M, in which lysine 1250 was changed to methionine) (Anderson et al., 1991*a*). These two stable cell lines were generated with the generous help of Dr. S. Thompson and Dr. R. C. Mulligan (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). We also transiently expressed another mutant (CFTR-K335E, in which lysine 335 in the first transmembrane domain was changed to glutamic acid) in HeLa cells using the vaccinia virus–T7 hybrid expression system (Rich et al., 1990; Elroy-Stein, Fuerst, and Moss, 1989). CFTR, in which part of the R domain was deleted (amino acids 708–835; CFTRΔR) (Rich et al., 1991), was stably expressed in a mouse mammary cell line (C127 cells). This stable C127 cell line was generated by first inserting CFTRΔR cDNA in place of wild-type CFTR cDNA in a bovine papilloma virus (BPV)–based expression vector. The CFTRΔR plasmid was then introduced into C127 cells using calcium phosphate and transfectants were selected by neomycin resistance. This stable

C127 cell line was a generous gift of Dr. R. J. Gregory and Dr. A. E. Smith (Genzyme Corp., Framingham, MA). Cells were maintained in culture as previously described (Rich et al., 1990).

Patch pipettes were back-filled with an intracellular solution containing (mM): 120 *N*-methyl-D-glucamine, 85 aspartic acid, 3 $MgCl_2$, 1 CsEGTA (ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, cesium salt; Sigma Chemical Co., St. Louis, MO), 1 MgATP, and 5 TES (*N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid), and pH was adjusted to 7.3 with HCl, yielding a Cl^- concentration of 43 mM. By replacing K^+ in the intracellular solution with the impermeant cation *N*-methyl-D-glucamine, K^+-selective currents were inhibited. The free Ca^{2+} concentration of the intracellular solution was $< 10^{-8}$ M.

Cells were washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and briefly treated with 0.25% (wt/vol) trypsin to detach them from the plastic Petri dishes on which they were grown.

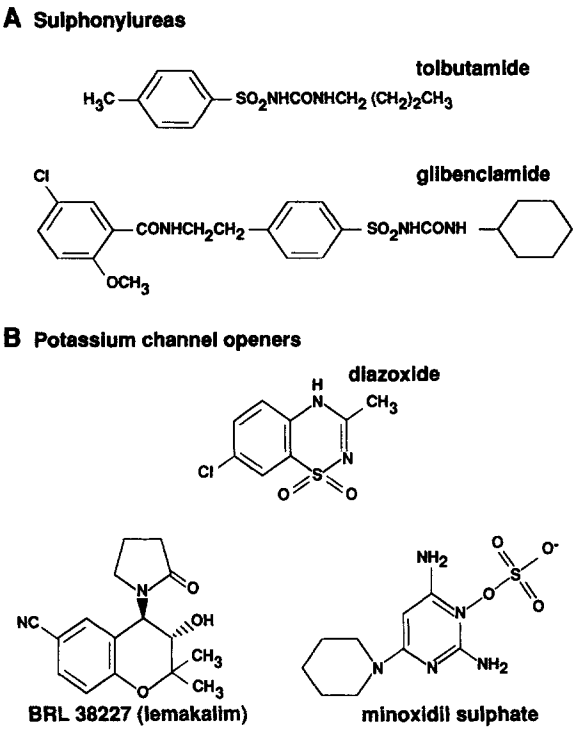


FIGURE 1. Structure of the sulfonylureas and K^+ channel openers used in this study.

This treatment did not affect the properties of CFTR Cl^- currents. Small aliquots of cells were placed in a chamber (volume = 0.5 cm^3) mounted on the stage of an inverted microscope and bathed in an extracellular solution containing (mM): 140 NaCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 10 dextrose, and 10 TES (pH 7.3 with NaOH). Test solutions were gently perfused into the bath and removed by a vacuum pump. All solutions were filtered through 0.45-μm filters (Millipore Corp., Bedford, MA) and experiments were conducted at 34–36°C using a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL).

Patch pipettes were fabricated from thin-walled borosilicate glass capillary tubing (Rochester Scientific Co., Rochester, NY) using a two-stage vertical pipette puller (model 750; David Kopf Instruments, Tujunga, CA) and coated with Sylgard (Dow Corning Corp., Midland, MI). Pipette tips were polished using a microforge. Patch pipettes had resistances of 2–4 MΩ when filled with intracellular solution.

Whole-cell membrane currents were recorded according to the method of Hamill, Marty, Neher, Sakmann, and Sigworth (1981) using a List EPC-7 amplifier (Adams and List Associates, Ltd., Westbury, NY). Seals of 10–40 GΩ were routinely obtained. Cells were clamped at a holding potential of 0 mV and membrane currents were measured during depolarizing and hyperpolarizing voltage steps.

The established sign convention was used throughout. That is, ionic currents produced by positive charge moving from intra- to extracellular solutions (anions moving in the opposite direction) are shown as positive currents. The bath electrode consisted of a Ag-AgCl pellet connected to the bathing solution via an agar bridge filled with 1 M KCl.

A microcomputer (IBM AT compatible) and the pClamp software package (Axon Instruments, Inc., Foster City, CA) were used for pulse generation, data acquisition, and analysis. Voltage-pulse protocols were applied to the stimulus input of the List EPC-7 amplifier after digital-to-analog conversion using a TL-1 DMA interface (Axon Instruments, Inc.). The signal from the patch-clamp amplifier, which was filtered at 0.5–2.5 kHz using a variable 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA), was simultaneously viewed on a storage oscilloscope and acquired using pClamp software.

Data shown have not been capacitance or leak subtracted. Results are expressed as means ± SEM of n observations.

Adenosine trisphosphate magnesium salt (MgATP), 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate sodium salt (CPT-cAMP), forskolin, 3-isobutyl-1-methylxanthine (IBMX), and TES were purchased from Sigma Chemical Co.

The chemical structures of the agents studied are shown in Fig. 1; they include tolbutamide (Sigma Chemical Co.), glibenclamide (Sigma Chemical Co.), diazoxide (Research Biochemicals Inc., Natick, MA), BRL 38227 (lemakalim, the biologically active enantiomer of cromakalim [Edwards and Weston, 1990], a generous gift of SmithKline Beecham Pharmaceuticals, Betchworth, UK), and minoxidil sulphate (a generous gift of the Upjohn Company, Kalamazoo, MI). Stock solutions of the sulfonylureas and diazoxide were prepared in dimethyl sulfoxide and BRL 38227 in 70% (vol/vol) ethanol; minoxidil sulfate was prepared in acetone according to the manufacturer's instructions. Drugs were diluted in extracellular solution to achieve final concentrations at the time of use. The vehicle solutions did not affect CFTR Cl^- currents ($n = 4$ in each case).

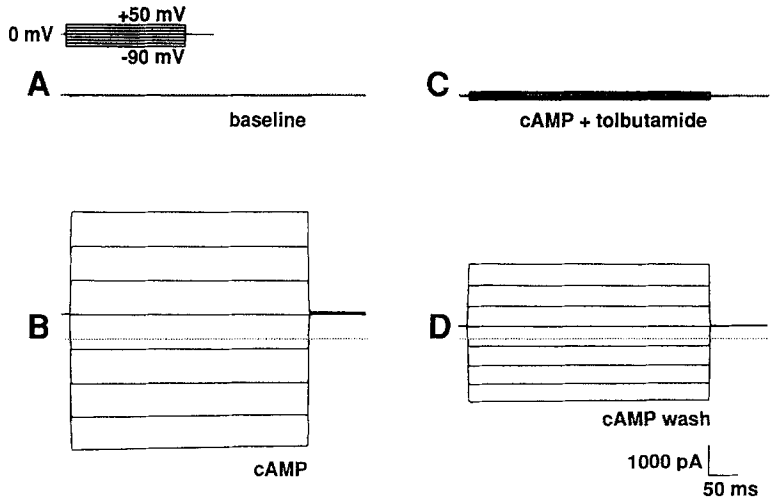
RESULTS

Cells expressing CFTR exhibited little or no Cl^--selective current under baseline conditions (Fig. 2 *A*). Addition of cAMP agonists (10 μM forskolin and 100 μM IBMX, or 500 μM CPT-cAMP, a membrane-permeant cAMP analogue) activated large Cl^--selective currents (Fig. 2 *B*). The current–voltage (*I-V*) relationship of the cAMP-regulated Cl^- current was linear (Fig. 2 *E*) and the currents exhibited no evidence of voltage-dependent activation or inactivation. In preliminary experiments, CFTR Cl^- current levels were stable over 3–5 min after activation.

Sulphonylurea Drugs

We examined the effect of the sulfonylurea drugs tolbutamide and glibenclamide by adding them after currents were activated by cAMP agonists. Fig. 2 *C* shows that 500 μM tolbutamide inhibited CFTR Cl^- currents. Inhibition of CFTR Cl^- currents showed little voltage dependence (Fig. 2, *C* and *E*).

Glibenclamide inhibits K-ATP channels more potently than tolbutamide (Zünkler, Lenzen, Männer, Panten, and Trube, 1988*a*). Fig. 3 *C* shows that 25 μM glibencla-



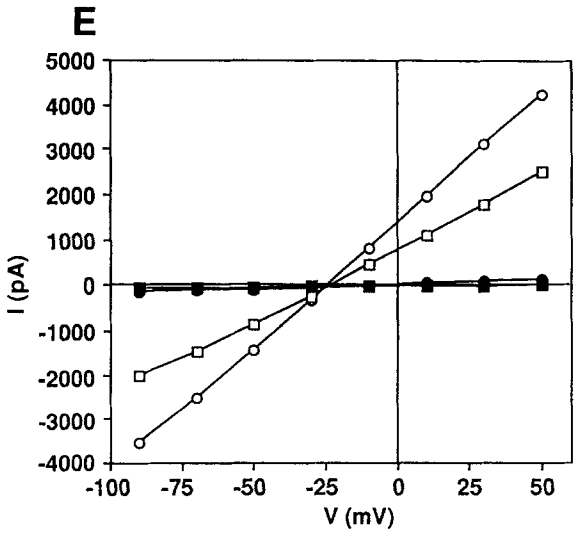


FIGURE 2. Tolbutamide inhibits CFTR Cl^- currents. Whole-cell currents were recorded from an NIH 3T3 fibroblast expressing CFTR. The inset shows that the holding potential was 0 mV and voltage was stepped from +50 to −90 mV in 20-mV decrements. Dotted line represents the zero current level. (*A*) Baseline conditions. (*B*) Currents recorded 2 min after adding 10 μM forskolin and 100 μM IBMX (cAMP). (*C*) Currents recorded 3 min after adding 500 μM tolbutamide. (*D*) Recovery of currents 15 min after washing the drug from the bath. (*E*) *I-V* relationship of currents inhibited by tolbutamide; current was measured at the end of a 400-ms voltage step. Data in *E* are from the traces in *A–D*. ■, baseline; ○, cAMP; ●, cAMP + tolbutamide; □, cAMP wash.

mide also potently inhibited CFTR Cl^- currents; inhibition showed little voltage dependence.

Inhibition by both sulfonylureas developed slowly; 3 min after adding 1 mM tolbutamide or 100 μM glibenclamide, CFTR Cl^- currents were inhibited by >90%. A second slower phase of current decay was also observed (not shown). This probably represents current "rundown", a phenomenon that has been previously documented for CFTR Cl^- channels (Berger et al., 1991). CFTR Cl^- currents partially recovered from tolbutamide treatment with washes lasting >10 min (Fig. 2 *D*). However, glibenclamide inhibition was irreversible even upon prolonged washing (Fig. 3 *D*).

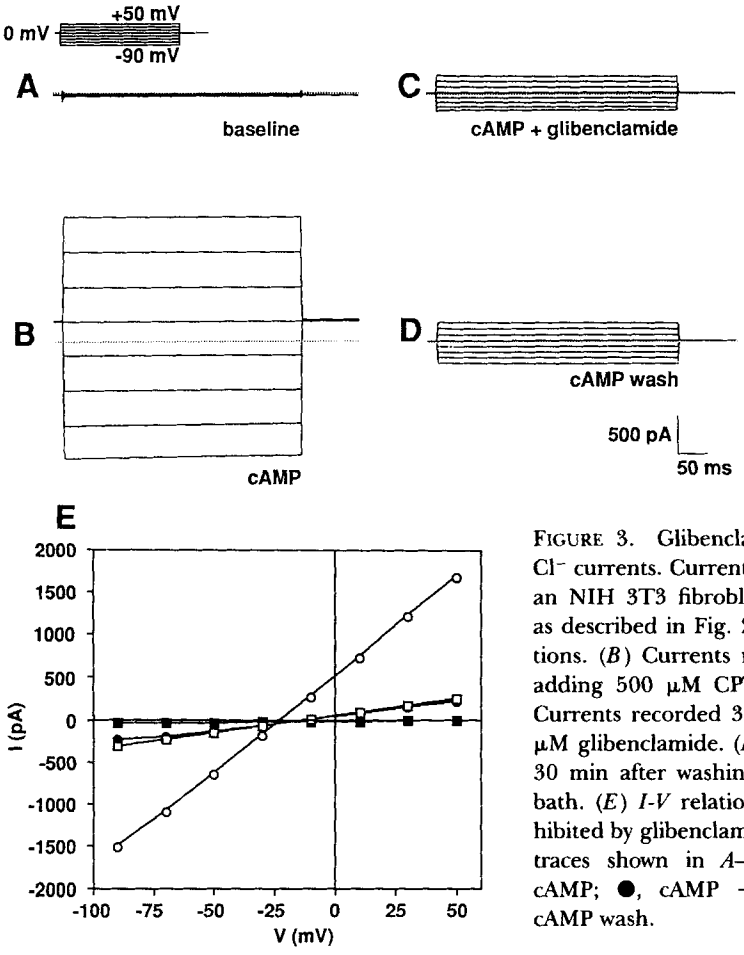


FIGURE 3. Glibenclamide inhibits CFTR Cl^- currents. Currents were recorded from an NIH 3T3 fibroblast expressing CFTR as described in Fig. 2. (*A*) Baseline conditions. (*B*) Currents recorded 2 min after adding 500 μM CPT-cAMP (cAMP). (*C*) Currents recorded 3 min after adding 25 μM glibenclamide. (*D*) Currents recorded 30 min after washing the drug from the bath. (*E*) *I-V* relationship of currents inhibited by glibenclamide; data are from the traces shown in *A–D*. ■, baseline; ○, cAMP; ●, cAMP + glibenclamide; □, cAMP wash.

Sulfonylurea inhibition was concentration dependent (Fig. 4). The relationship between drug concentration and current inhibition was fitted to the equation:

$$I\ (\%\ \text{control}) = 1/\{1 + ([\text{drug}]/K_i)^n\} \quad (1)$$

where K_i is the drug concentration (micromolar) causing half-maximal inhibition and n is the Hill coefficient. Glibenclamide was more potent than tolbutamide and for both drugs n was < 1 (Table I).

Potassium Channel Openers

ATP-regulated K^+ channels are activated by a family of drugs called K^+ channel openers, which include the compounds diazoxide, BRL 38227, and minoxidil sulfate (Standen et al., 1989; Edwards and Weston, 1990). We tested whether K^+ channel openers could stimulate CFTR Cl^- channels in NIH 3T3 fibroblasts stably expressing CFTR. Diazoxide (100–1,000 μM), BRL 38227 (250 μM), and minoxidil sulfate (10–500 μM) had no effect on baseline currents ($n = 3$ in each case; results not

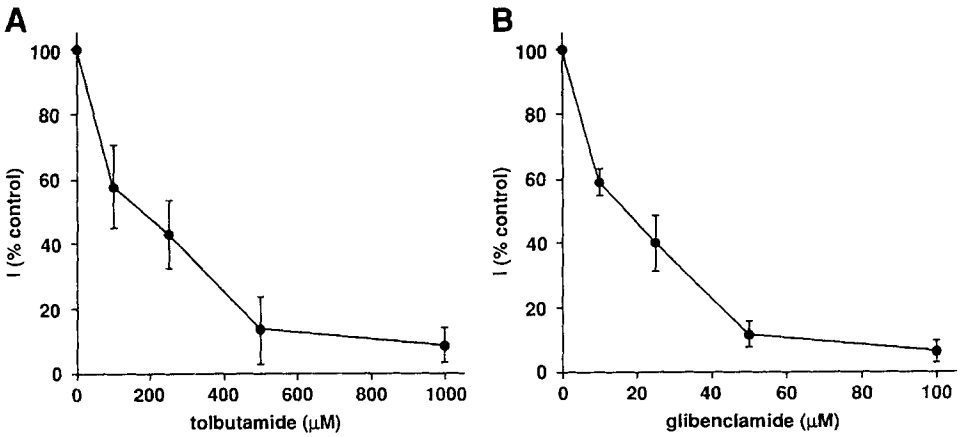


FIGURE 4. Dose–response curves for the effect of sulfonylureas on CFTR Cl^- currents. Data are from currents recorded 3 min after adding increasing concentrations of tolbutamide (*A*) and glibenclamide (*B*). Currents were measured at the end of 400-ms voltage steps to +50 mV and are expressed as percent control current. Baseline currents, which measured $< \pm 100$ pA at +50 and −90 mV, have not been subtracted. Results represent the mean ± SEM of 5–12 observations at each concentration.

shown). However, the subsequent activation of CFTR Cl^- currents by cAMP-mediated agonists was consistently attenuated in the presence of K^+ channel openers when compared with that normally observed upon cAMP stimulation (not shown). Because of this result, we examined the effect of K^+ channel openers on CFTR Cl^- currents that had previously been activated by cAMP agonists.

After CFTR Cl^- currents were activated by cAMP agonists (Fig. 5, *A*, *D*, and *G*) we added either diazoxide, BRL 38227, or minoxidil sulfate to the bathing solution (Fig. 5, *B*, *E*, and *H*). In each case, Cl^- current was inhibited. Inhibition showed little voltage dependence. As was observed with the sulfonylureas, inhibition by the K^+ channel openers developed slowly; 3 min after adding 1 mM diazoxide, 250 μM BRL 38227, or 100 μM minoxidil sulfate CFTR Cl^- currents were inhibited by $\geq 70\%$. A

TABLE I

Effect of Sulfonylureas and K^+ Channel Openers on CFTR Cl^- Currents

Drug	K_i	n	Experiments
	μM		
Tolbutamide	149.5 ± 80.83	0.8 ± 0.1	5
Glibenclamide	21.8 ± 5.2	0.8 ± 0.1	7
Diazoxide	259.9 ± 51.0	0.6 ± 0.1	5
BRL 38227	48.3 ± 4.5	0.8 ± 0.2	4
Minoxidil sulfate	37.9 ± 9.6	0.5 ± 0.1	4

Data from individual experiments were fitted to a linear form of Eq. 1 using a linear least-squares regression program to yield drug concentration causing half-maximal inhibition, K_i, and Hill coefficients, n. The number of individual experiments is indicated. From these data the tabulated mean values were derived. The results were calculated from currents measured at +50 mV without subtraction of residual current.

second slower phase of current decay was also observed (not shown) which probably represents current "rundown". Inhibition was poorly reversible over 5-min time intervals (not shown); longer washes were not tested.

Inhibition by K^+ channel openers was concentration dependent (Fig. 6). The relationship between drug concentration and current inhibition was fitted to Eq. 1

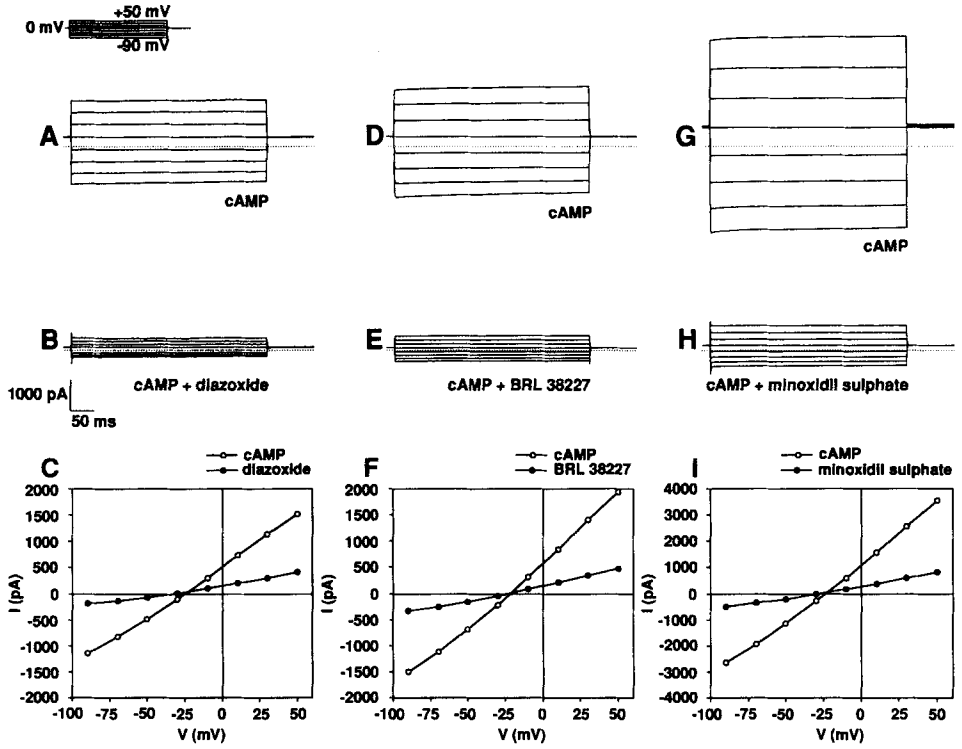


FIGURE 5. K^+ channel openers inhibit CFTR Cl^- currents. Traces are from NIH 3T3 fibroblasts expressing CFTR as described in Fig. 2. Under baseline conditions, membrane currents measured $< \pm 100$ pA at +50 and −90 mV. *A*, *D*, and *G* show CFTR Cl^- currents recorded 2 min after addition of cAMP agonists. *B*, *E*, and *H* show currents recorded 3 min after the addition of 500 μM diazoxide (*B*), 100 μM BRL 38227 (*E*), and 100 μM minoxidil sulfate (*H*). The corresponding *I-V* relationships are shown in *C*, *F*, and *I*. Currents were measured as described in Fig. 2.

and the results are shown in Table I. Minoxidil sulfate was more potent than BRL 38227, which was more potent than diazoxide. In each case n was < 1.

Effect of Glibenclamide on CFTR Mutants

We were interested to learn how sulfonylureas and K^+ channel openers interact with CFTR. We therefore examined the effect of glibenclamide, the most potent inhibitor we had identified, on Cl^- currents generated by several CFTR mutants. We studied

CFTR containing mutations that affect each of the three types of domains of CFTR: CFTRΔR where part of the R domain has been deleted (amino acids 708–835); CFTR-K335E, which contains a mutation in the sixth putative membrane-spanning sequence; and CFTR-K1250M, which contains a mutation in the second NBD. We thought it possible that if glibenclamide specifically interacts with one of these domains, its inhibitory properties might be altered.

CFTRΔR forms Cl^- channels that are active independent of cAMP stimulation (Rich et al., 1991). This is illustrated in Fig. 7 *A*,which shows a family of CFTRΔR Cl^- currents recorded in the absence of cAMP agonists (as in all previous studies, the pipette contained 1 mM MgATP). Although CFTRΔR Cl^- channels are constitutively active, they possess the same biophysical properties as wild-type CFTR Cl^- channels: linear *I-V* relationship, no evidence of voltage-dependent activation or inactivation, small single-channel conductance, selectivity for anions over cations, and an anion permeability sequence of $Br^- > Cl^- > I^-$ (see Rich et al., 1991; Anderson et al.,

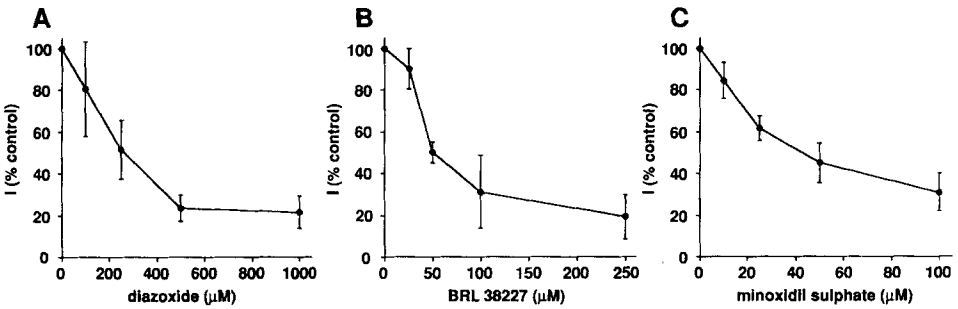


FIGURE 6. Dose–response curves for the effect of K^+ channel openers on CFTR Cl^- currents. Data are for diazoxide (*A*), BRL 38227 (*B*), and minoxidil sulfate (*C*), measured as described in Fig. 4, and are expressed as percent control current. Baseline currents, which measured $< \pm 100$ pA at +50 and −90 mV, have not been subtracted. Results represent the mean ± SEM of four to eight observations at each concentration.

1991*a*; and Fig. 7, *A* and *C*). The small, slowly activating and inactivating current components observed at large positive and negative potentials (Fig. 7 *A*) were observed infrequently, and are also observed in some cells expressing wild-type CFTR (Anderson et al., 1991*b*; Rich et al., 1991).

Although deletion of the R domain did not prevent inhibition by glibenclamide, its inhibitory properties differed from those observed with wild-type CFTR. Inhibition was significantly more potent at hyperpolarizing voltages than at equivalent depolarizing voltages (Fig. 7, *B* and *C*).

CFTR-K335E forms Cl^- channels that are similar to wild-type channels, except that the anion selectivity is altered such that $I^- > Br^- > Cl^-$ and currents show some outward rectification (Anderson et al., 1991*b* and Fig. 7, *D* and *F*). Glibenclamide inhibited CFTR-K335E Cl^- currents, and the effect was similar to that observed with wild-type CFTR (Fig. 7, *E* and *F*).

Comparison with ATP-dependent transporters suggest that mutations in the second NBD, such as CFTR-K1250M, are likely to abolish or impair the function of

NBD2 (Anderson et al., 1991*a*). Nevertheless, CFTR-K1250M produces Cl^- channels that have cAMP-dependent regulation and biophysical properties that are similar to wild-type CFTR (see Anderson et al., 1991*a*, and Fig. 7, *G* and *I*). Inhibition of CFTR-K1250M Cl^- currents by glibenclamide was similar to that observed with wild-type CFTR (Fig. 7, *H* and *I*).

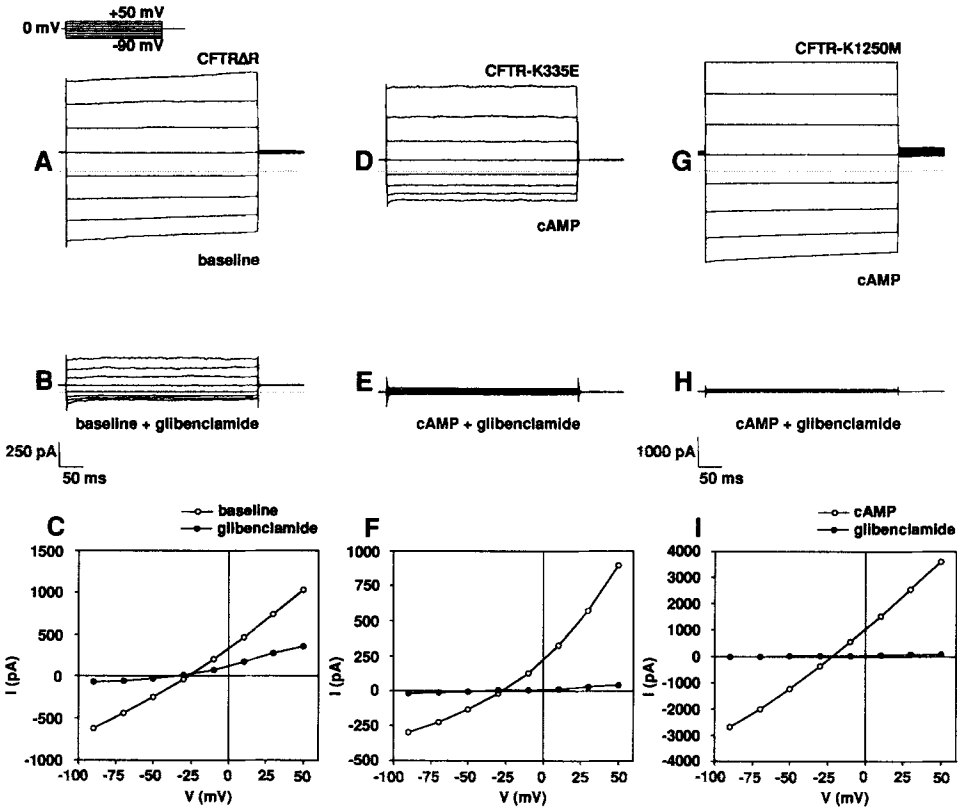


FIGURE 7. Glibenclamide inhibits CFTR mutants. Traces are from C127 cells stably expressing CFTRΔR (*A–C*), HeLa cells transiently expressing CFTR-K335E (*D–F*), and NIH 3T3 cells stably expressing CFTR-K1250M (*G–I*). Currents were measured as described in Fig. 2. CFTRΔR currents were recorded in the absence of cAMP, but for CFTR-K335E and CFTR-K1250M currents cAMP agonists were present. Before addition of cAMP agonists, currents measured $< \pm 100$ pA at +50 and −90 mV. *B*, *E*, and *H* show currents recorded 3 min after adding 100 μM glibenclamide. The corresponding *I-V* relationships of CFTR mutants inhibited by glibenclamide are shown in *C*, *F*, and *I*. Currents were measured as described in Fig. 2.

Fig. 8 shows the relationship between glibenclamide concentration and current produced by wild-type and CFTR mutants. Data for steady-state current values measured at +50 and −90 mV are shown for wild-type CFTR (*A*), CFTRΔR (*B*), CFTR-K335E (*C*), and CFTR-K1250M (*D*). The dose–response curves for wild-type CFTR, CFTR-K335E, and CFTR-K1250M were similar. Although the effect was not marked, in each case inhibition was slightly more potent at −90 mV than at +50 mV.

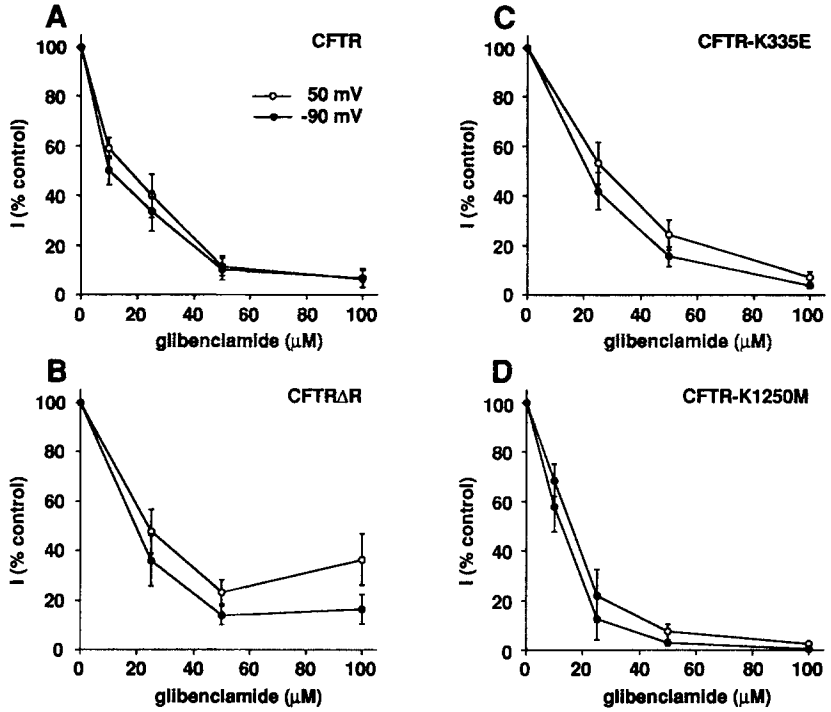


FIGURE 8. Dose–response curves for inhibition of CFTR mutants by glibenclamide. Data were obtained and expressed as described in Fig. 4. Baseline currents, which measured $< \pm 100$ pA at +50 and −90 mV, have not been subtracted. (*A*) Wild-type CFTR; (*B*) CFTRΔR; (*C*) CFTR-K335E; (*D*) CFTR-K1250M. Results represent the mean ± SEM of 3–11 observations at each concentration.

With CFTRΔR, glibenclamide was substantially more potent at hyperpolarizing (−90 mV) than at depolarizing (+50 mV) voltages ($P < 0.05$ at 100 μM glibenclamide). In addition, 100 μM glibenclamide inhibited less current at +50 mV in CFTRΔR than in wild-type CFTR ($P = 0.005$).

The data for all the mutants except CFTRΔR were well fitted by Eq. 1 (Table II). Half-maximal inhibition by glibenclamide was similar in all cases, occurring at ~20

TABLE II

Effect of Glibenclamide on CFTR Mutants

Mutant	+50 mV		−90 mV		Experiments
	K_i	n	K_i	n	
	μM		μM		
CFTR	21.8 ± 5.2	0.8 ± 0.1	19.0 ± 5.6*	0.7 ± 0.1	7
CFTR-K335E	25.9 ± 4.1	0.9 ± 0.1	20.4 ± 3.7*	0.9 ± 0.1	6
CFTR-K1250M	16.8 ± 4.2	0.8 ± 0.1	31.2 ± 2.5	1.0 ± 0.1	5

K_i and n values were calculated as described in Table I for currents measured at +50 and −90 mV for the number of experiments listed. Asterisks indicate statistically significant difference between K_i values ($P < 0.05$).

μM; *n* values were also comparable and less than 1. Half-maximal inhibition by glibenclamide, measured at −90 mV, showed a small but statistically significant increase in potency compared with that at +50 mV for wild-type CFTR and CFTR-K335E, but not CFTR-K1250M.

DISCUSSION

The goal of this study was to find modulators of CFTR Cl^- channels. The data demonstrate that agents that regulate the opening of K-ATP channels also inhibit CFTR Cl^- channels with a rank order of potency of: glibenclamide > minoxidil sulfate ≅ BRL 38227 > tolbutamide > diazoxide. Thus, these agents have a wider specificity of action than previously recognized. Glibenclamide represents a potent blocker of the CFTR Cl^- channel.

Comparison of K-ATP Channels and CFTR Cl^- channels

Our study of agents that modulate K-ATP channel activity was prompted by similarities between K-ATP channels and CFTR. K-ATP channels have not yet been sequenced and probably represent a heterogeneous class of K^+ channels since there are tissue-specific differences in their biophysical properties and regulation (Ashcroft and Ashcroft, 1990). Nevertheless, K-ATP channels share some functional properties with CFTR Cl^- channels. Both CFTR and K-ATP channels can be regulated by PKA-dependent phosphorylation (Ribalet, Ciani, and Eddlestone, 1989; Berger et al., 1991; Tabcharani et al., 1991). In addition, both channels are relatively insensitive to changes in membrane potential and to the intracellular free Ca^{2+} concentration (Cook and Hales, 1984; Anderson et al., 1992). The most notable similarity between these channels is regulation by intracellular ATP. The effect of ATP, however, differs for the two types of channel: ATP activates CFTR Cl^- channels, whereas it inhibits K-ATP channels (Noma, 1983; Cook and Hales, 1984; Anderson et al., 1991*a*). Another difference is that hydrolyzable nucleoside triphosphates are required to regulate CFTR, whereas nonhydrolyzable analogues of ATP inhibit K-ATP channels (Dunne, West-Jordan, Abraham, Edwards, and Petersen, 1988; Anderson et al., 1991*a*).

The modulation of K-ATP channels in excitable cells by intracellular ATP couples cellular metabolism and electrical activity. For example, K-ATP channels establish the resting membrane potential in pancreatic β cells and their closure by glucose metabolites initiates insulin secretion (see Dunne and Petersen, 1991, for review). In contrast, in cardiac myocytes K-ATP channels are normally quiescent but they activate during ischemia to increase K^+ efflux and decrease the action potential duration (Gasser and Vaughan-Jones, 1990). These effects are believed to be important in preserving cellular homeostasis. It has been speculated that CFTR Cl^- channels fulfill a similar homeostatic mechanism in epithelia (Quinton, 1990). Regulation of CFTR Cl^- channels by intracellular ATP may represent a strategy by which secretory epithelial cells balance the rate of transepithelial Cl^- secretion with cellular ATP levels, thereby controlling cell volume and ionic composition (Quinton, 1990; Anderson et al., 1991*a*).

Effect of K^+ Channel Regulators on CFTR Cl^- Channels

Tolbutamide and glibenclamide inhibit K-ATP channels in pancreatic β cells with half-maximal inhibition at ~4 μM and 4 nM, respectively (Zünkler et al., 1988*a*). However, in cardiac myocytes, higher concentrations of sulfonylureas are required to block K-ATP channels: Belles, Hescheler, and Trube (1987) reported half-maximal inhibition of whole-cell K-ATP currents at >300 μM tolbutamide. The sulfonylureas tolbutamide and glibenclamide inhibited CFTR Cl^- currents at half-maximal concentrations of ~150 and 20 μM, respectively. Inhibition showed little voltage dependence, developed slowly, and was reversible with tolbutamide but not with glibenclamide. These characteristics are similar to the effect of sulfonylureas on K-ATP channels (Belles et al., 1987; Gillis, Gee, Hammoud, McDaniel, Falke, and Misler, 1989).

In contrast to their effect on K-ATP channels, diazoxide, BRL 38227, and minoxidil sulfate did not stimulate CFTR Cl^- channels, either under baseline or cAMP-stimulated conditions. Instead, these agents inhibited CFTR Cl^- channels. As observed with the sulfonylureas, blockade showed little voltage dependence, developed slowly, and was poorly reversible. The potency of K^+ channel openers for inhibition of CFTR Cl^- channels (minoxidil sulfate [40 μM] ≅ BRL 38227 [50 μM] > diazoxide [250 μM]) was weaker than their effect on K-ATP channels in smooth muscle. In vascular smooth muscle, 1 μM cromakalim (Standen et al., 1989) and 2 μM minoxidil sulfate (Leblanc, Wilde, Keef, and Hume, 1989) activate K-ATP channels, and in pancreatic β cells 100 μM diazoxide (Dunne, Aspinall, and Petersen, 1990) stimulates K-ATP channels. However, there is marked tissue specificity in the action of K^+ channel openers; for example, 800 μM cromakalim is needed to match the activation of K-ATP channels by 100 μM diazoxide in pancreatic β cells (Dunne et al., 1990).

The activation of K-ATP channels by K^+ channel openers is dependent on the presence of cytosolic factors. In the absence of intracellular ATP, diazoxide and cromakalim are ineffective. However, high concentrations of ATP are also inhibitory, suggesting a competitive interaction between ATP and K^+ channel openers (Kozlowski, Hales, and Ashford, 1989; Dunne et al., 1990). Diazoxide is also ineffective in the absence of intracellular Mg^{2+} (Kozlowski et al., 1989). The activation of K-ATP channels only occurs in the presence of cytosolic MgATP; in its absence K^+ channel openers are either without effect or inhibit K-ATP channels (Kozlowski et al., 1989; Dunne, 1990). Interestingly, diazoxide blocks cardiac K-ATP channels regardless of whether or not MgATP is present (Faivre and Findlay, 1989). K^+ channel openers are also ineffective when nonhydrolyzable ATP analogues are substituted for ATP, suggesting that the drugs may act on a phosphorylated form of the channel (Kozlowski et al., 1989; Dunne et al., 1990).

Sulfonylureas are highly lipid soluble, suggesting that they may permeate the plasma membrane to exert their effect. This idea is supported by the finding that sulfonylureas are equally effective in inhibiting K-ATP channels when applied to the intra- or extracellular membrane surface (Belles et al., 1987; Gillis et al., 1989). In addition, increasing extracellular pH slows the onset of tolbutamide inhibition of K-ATP channels, suggesting that the undissociated form of the drug is the active

moiety (Zünkler, Trube, and Panten, 1989). The slow onset of sulfonylurea inhibition of CFTR Cl^- currents is consistent with the idea that the drugs permeate the cell membrane to exert their effect.

The action of sulfonylureas on K-ATP channels is also regulated by intracellular compounds. Cytosolic ADP increases the tolbutamide sensitivity of K-ATP channels in pancreatic β cells, while other nucleotides are without effect (Zünkler, Lins, Ohno-Shosaku, Trube, and Panten, 1988*b*). However, in cardiac myocytes the converse effect is seen; ADP attenuates glibenclamide inhibition of K-ATP channels (Venkatesh and Weiss, 1991). During whole-cell recordings ATP in the pipette-filling solution may be hydrolyzed to ADP by enzymes remaining in the cell. It is therefore possible that the K_D values for CFTR Cl^- current inhibition by sulfonylureas and K^+ channel openers were influenced by the composition of the pipette-filling solution. The Hill coefficient for inhibition of CFTR Cl^- currents was < 1 for all the drugs tested. This suggests that cooperativity is not important for the interaction of these agents with CFTR. Similar values of Hill coefficients have been reported for the effect of tolbutamide and diazoxide on K-ATP channels, although higher values were reported for glibenclamide (Zünkler et al., 1988*a*).

In an attempt to gain some insight into how these agents might interact with CFTR, we examined the effect of glibenclamide, the most potent inhibitor we had identified, on Cl^- currents generated by several CFTR mutants. We chose mutations in each of the three types of domains of CFTR. Inhibition of CFTR-K335E and CFTR-K1250M Cl^- currents by glibenclamide resembled that of wild-type CFTR. This suggests that residues K335E and K1250M do not form a critical part of the glibenclamide interaction site. Nevertheless, other residues within the membrane-spanning and nucleotide-binding domains may contribute to the interaction.

Glibenclamide also inhibited CFTRΔR Cl^- currents, but the inhibition showed significant voltage dependence, with a greater potency at hyperpolarizing voltages. This result suggests that the R domain in some way affects the response to glibenclamide such that deletion of part of the R domain partially relieves the block of CFTR Cl^- currents by glibenclamide at depolarized voltages. However, the data also indicate that the major part of the R domain is not required for glibenclamide interaction. At present we do not have an explanation for this interesting observation.

Glibenclamide represents a potent inhibitor of CFTR Cl^- currents. In comparison, millimolar concentrations of extracellular diphenylamine-2-carboxylate (DPC) are required to block CFTR Cl^- currents by ~80% (Anderson et al., 1991*c*, 1992; McCarty, Cohen, Quick, Riordan, Davidson, and Lester, 1992); the arylaminobenzoate NPPB (100–200 μM) applied extracellularly is reported to be less efficient than DPC (McCarty et al., 1992). Inhibition by DPC is voltage dependent with stronger blockade at negative potentials, suggesting that it interacts with the channel from the intracellular surface (McCarty et al., 1992). Extracellular DIDS, a stilbene–disulfonic acid derivative that blocks several types of epithelial Cl^- channels does not affect CFTR Cl^- currents at concentrations of 500 μM (Kartner et al., 1991; Anderson et al., 1992). Similarly, the Cl^- channel blockers Zn^{2+} and the indanyloxyacetic acid derivative IAA-94 are ineffective inhibitors when applied extracellularly at concentrations of 100 and 40 μM, respectively (Anderson et al., 1992). Thus, glibenclamide is the most potent inhibitor of CFTR identified to date.

Implications for Disease

One of our initial goals in this study was to find agents that would activate CFTR. We thought that the discovery of novel pharmacological activators of CFTR Cl^- channels might provide a new therapeutic strategy for treating CF patients. Although we did not find agents that activate CFTR, the finding that CFTR is inhibited by sulfonylureas and K^+ channel openers suggests an interaction and raises the possibility that related agents might prove to be valuable activators of CFTR. A similar relationship between channel activators and inhibitors exists with the dihydropyridine class of Ca^{2+} channel agonists and antagonists (Hess, Lansman, and Tsien, 1984).

It is, however, interesting to consider the possibility that these or related agents might be of value in another disease that probably involves CFTR Cl^- channels. CFTR is located within the apical membrane of Cl^--secreting intestinal epithelial cells (Crawford, Maloney, Zeitlin, Guggino, Hyde, Turley, Gatter, Harris, and Higgins, 1991; Denning, Ostedgaard, Cheng, Smith, and Welsh, 1992). Chloride efflux through CFTR Cl^- channels probably contributes to the watery diarrhea caused by microbial toxins such as cholera toxin and heat-stable *Escherichia coli* enterotoxin (Fondacaro, 1986). The development of therapeutically active blockers of CFTR Cl^- channels might therefore provide a treatment for some forms of diarrhea. The potency of glibenclamide inhibition of CFTR Cl^- channels suggests that it may be of value in the design and synthesis of such drugs.

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