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Antihypertensive 1,4-Dihydropyridines as Correctors of the Cystic Fibrosis Transmembrane Conductance Regulator Channel Gating Defect Caused by Cystic Fibrosis Mutations

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ABSTRACT

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel gene. CF mutations like ΔF508 cause both a mistrafficking of the protein and a gating defect. Other mutations, like G551D, cause only a gating defect. Our aim was to find chemical compounds able to stimulate the activity of CFTR mutant proteins by screening a library containing approved drugs. Two thousand compounds were tested on Fischer rat thyroid cells coexpressing ΔF508-CFTR and a halide-sensitive yellow fluorescent protein (YFP) after correction of the trafficking defect by low-temperature incubation. The YFP-based screening allowed the identification of the antihypertensive 1,4-dihydropyridines (DHPs) nifedipine, nicardipine, nimodipine, isradipine, nitrendipine, felodipine, and niguldipine as compounds able to activate ΔF508-CFTR. This effect was not derived from the inhibition of voltage-dependent Ca^{2+} channels, the pharmacological target of antihypertensive DHPs. Indeed, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4–2(trifluoromethylphenyl)pyridine-5-carboxylate (BayK-8644), a DHP that is effective as an activator of such channels, also stimulated CFTR activity. DHPs were also effective on the G551D-CFTR mutant by inducing a 16- to 45-fold increase of the CFTR Cl^- currents. DHP activity was confirmed in airway epithelial cells from patients with CF. DHPs may represent a novel class of therapeutic agents able to correct the defect caused by a set of CF mutations.

Cystic fibrosis (CF) is caused by mutations that impair the function of a cAMP-activated plasma membrane Cl^- channel termed CFTR (Sheppard and Welsh, 1999). CFTR is a member of the ATP-binding cassette transporter superfamily (Hyde et al., 1990) like P-glycoprotein (MDR-1) and multidrug resistance proteins and consists of five distinct domains: two membrane-spanning domains, two nucleotide-binding domains (NBDs), and a regulatory domain. ATP binding and possibly hydrolysis at the NBDs are responsible for channel gating, whereas cAMP-dependent phosphorylation of the regulatory domain regulates channel activity (Sheppard and Welsh, 1999). CF mutations have been grouped into five classes, depending on the mechanisms through which they cause the functional defect (Welsh and Smith, 1993). Class II mutations cause a mistrafficking of CFTR so that the protein remains mainly trapped in the endoplasmic reticulum and subsequently degraded. Conversely, class III mutations do not impair protein trafficking but severely decrease CFTR channel opening. The deletion of phenylalanine 508 (ΔF508), the most frequent CF mutation, causes both a trafficking (Denning et al., 1992; Lukacs et al., 1994) and a channel-opening defect (Dalemans et al., 1991; Haws et al., 1996), although the latter one is less severe than that of class III mutants. In fact, when the ΔF508-CFTR protein is induced to traffic to the plasma membrane by incubation at low temperature or by overexpression, it shows an open channel probability that is nearly 3-fold lower than that of the wild-type CFTR (Dalemans et al., 1991; Haws et al., 1996). A typical

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ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; YFP, yellow fluorescent protein; FRT, Fischer rat thyroid; HTS, high-throughput screening; DHP, 1,4-dihydropyridine; NBD, nucleotide binding domain; BayK-8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4–2(trifluoromethylphenyl)pyridine-5-carboxylate; PBS, phosphate-buffered saline; TES, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulfonic acid; MRS-1845, *N*-propargylnitrendipine.

example of pure class III mutation is instead represented by G551D (glycine-to-aspartic acid change at position 551), which causes a severe impairment in CFTR channel activity (Gregory et al., 1991; Logan et al., 1994; Zegarra-Moran et al., 2002). Various chemical compounds are known to stimulate the activity of CFTR mutants affected by altered channel gating. For example, genistein and other flavonoids, at high micromolar concentrations, strongly stimulate the activity of G551D and of ΔF508 (Hwang et al., 1997; Illek et al., 1999; Zegarra-Moran et al., 2002). Other compounds active on mutant CFTR include xanthines, benzoquinoliziniums salts, and benzimidazolones (Haws et al., 1996; Devor et al., 2000; Al-Nakkash et al., 2001; Derand et al., 2001). Novel classes of potent CFTR activators have been identified by high-throughput screening (HTS) of large chemical libraries (Ma et al., 2002b; Yang et al., 2003). Some of the new chemical entities identified by HTS or other strategies could be the starting point to develop drugs for the effective treatment of CF primary defect. However, a long and expensive series of preclinical studies that include the determination of specificity, toxicity, and pharmacokinetics are needed before undertaking clinical trials. As a strategy to find CFTR-modulating molecules with faster application in vivo, we decided to screen a library containing drugs that have already been approved for the treatment of other human diseases. Using an assay derived from the halide-sensitivity of yellow fluorescent proteins (YFPs) (Galietta et al., 2001a), we tested a collection of 2000 small molecules that include natural compounds.

We have found that the 1,4-dihydropyridines (DHPs) used to treat hypertension are able to stimulate the activity of ΔF508- and G551D-CFTR mutants. Antihypertensive DHPs work by blocking L-type voltage-dependent Ca^{2+} channels and therefore cause the relaxation of arterial smooth muscle cells (Harrold, 2002). Our experiments suggest that mutant CFTR is activated by DHPs through a mechanism not involving the modulation of Ca^{2+} channels, possibly by direct interaction with the CFTR protein itself. These findings may represent the basis for the development of new treatments for CF.

Materials and Methods

Cell Culture. Fischer rat thyroid (FRT) cells, stably transfected with ΔF508- or G551D-CFTR (Galietta et al., 2001b; Zegarra-Moran et al., 2002), were retransfected with the YFP-H148Q/I152L fluorescent protein, which allows optimal sensitivity for the detection of mutant CFTR activity (Galietta et al., 2001a). The cells were cultured on solid support in Coon's modified F-12 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For fluorescence assays, cells were plated (100,000 cells/well) on black 96-well microplates with clear plastic bottom (Corning Life Sciences, Acton, MA). For Ussing chamber experiments, FRT cells were seeded into Snapwell permeable supports (Corning Life Sciences) at 500,000 cells per insert. Polarized preparations of human airway epithelial cells were generated on the same type of Snapwell inserts as described previously (Galietta et al., 1998). In brief, nasal polyps from persons with and without CF were digested overnight with protease XIV to detach surface epithelial cells. Cells were initially grown in flasks using a serum-free medium that allowed cell proliferation. Thereafter, the cells were plated at high density on the permeable supports. At this stage, the medium was replaced with a mixture of Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 2% fetal calf serum and various hormones to favor cell differentiation (Galietta et al., 1998).

Compounds. The Spectrum library was purchased from MicroSource Discovery Systems (Gaylordsville, CT). The list of compounds included in the library is available at the MicroSource Discovery web site (http://www.msdiscovery.com/spect.html). Compounds were obtained as 10 mM stock solutions in dimethyl sulfoxide. Daughter plates were made at 1 mM concentration in dimethyl sulfoxide using a Biomek 2000 liquid handling workstation (Beckman Coulter, Fullerton, CA). All plates were stored at −80°C.

The powders for MRS-1845, isradipine, niguldipine enantiomers, and BayK-8644 enantiomers were purchased from Tocris Cookson Inc. (Ellisville, MO). The other DHPs and all other reagents were from Sigma-Aldrich (St. Louis, MO).

Separation of Isradipine Enantiomers. The high-performance liquid chromatography separation was performed on a BioCAD 700E Perseptive Biosystems (Applied Biosystems, Foster City, CA). The column was a direct-phase Chiralcel OJ-H (Daicel Chemical Industries, Osaka, Japan), 250 × 4.6 mm, 5 μm, and flow rate of 1.5 ml/min. The volume of loop was 100 μl. The UV detector was set at 254 nm. The separation was performed in isocratic conditions with *i*-propanol/*n*-hexane (1:9) as eluent.

Twenty milligrams of (±)-isradipine was dissolved in 2 ml of *i*-propanol. This solution (100 μl) was injected into the chromatograph, and this operation was repeated until all solution passed through the chromatograph. The chromatogram showed two peaks: peak A (reaction time, 9.0 min; area, 48%), peak B (reaction time, 12.0 min; area, 52%). All of the eluates of peak A were collected together, and the solvent was evaporated under reduced pressure, obtaining a yellowish solid. The same procedure was done for peak B. The two solids (A and B) were dissolved in a small quantity of diethyl ether, precipitated with *n*-hexane, and filtered off.

For optical rotation analysis, the solid A and solid B were dissolved separately in 1 ml of ethanol and poured in a 1-ml cell of a polarimeter at room temperature (Polarimeter 241; PerkinElmer Life and Analytical Sciences, Boston, MA) using a 599-nm source obtained from a sodium lamp. Solid A was "right-handed" [(+)-isradipine] and solid B was "left-handed" [(−)-isradipine].

Extraction of Amlodipine. Tablets of the pharmaceutical preparation containing amlodipine were ground and treated with 20 ml of chloroform. The resulting mixture was filtered, and the precipitate was crystallized from ethanol. The resulting white solid melted at 198 to 200°C. The thin-layer chromatography analysis revealed the presence of a single spot. The NMR spectrum confirmed the structure of amlodipine.

Extraction of Lercanidipine. Tablets of the pharmaceutical preparation containing lercanidipine were ground and treated with 20 ml of water. The mixture was treated with saturated sodium bicarbonate solution and extracted with chloroform. The organic phase was dried with anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The yellow solid so obtained was crystallized from petroleum ether. The filtered solid melted at 99 to 100°C. The thin-layer chromatography analysis revealed the presence of a single spot. The NMR spectrum confirmed the structure of lercanidipine.

Fluorescence Assay. The 96-well microplates containing FRT cells expressing ΔF508-CFTR and the halide-sensitive YFP were incubated at 27°C for 20 to 24 h to allow rescue of the mutant protein to the plasma membrane. After incubation, cells were washed with PBS (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and stimulated for 20 min with forskolin and test compounds in a final volume of 60 μl. Microplates were then transferred to a microplate reader (FluoStar Galaxy; BMG Labtech GmbH, Offenburg, Germany) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for yellow fluorescent protein (Chroma Technology Corp., Brattleboro, VT). Each assay consisted of a continuous

14-s fluorescence reading (5 points per second) with 2 s before and 12 s after injection of 165 μl of an iodide-containing solution (PBS with Cl^- replaced by I^-). Final iodide concentration in the wells was 100 mM. These data were normalized to the initial background-subtracted fluorescence. To determine I^- influx rate, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope.

cAMP Assay. Intracellular cAMP concentration was measured using the BIOTRAK enzymatic immunoassay (GE Healthcare, Little Chalfont, Buckinghamshire, UK). FRT cells expressing ΔF508-CFTR were cultured in 96-well plates. After washing with PBS, cells were incubated for 15 min with compounds in the presence of a submaximal concentration of forskolin (0.5 μM) and then lysed. Lysates were assayed for cAMP content in triplicate according to the manufacturer's instructions.

Transepithelial Current Measurements. Snapwell inserts were mounted in a self-contained Ussing chamber system (Vertical diffusion chamber; Corning Life Sciences). FRT cells were studied with a transepithelial Cl^- gradient. Therefore, the basolateral solution contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM sodium HEPES, pH 7.3, and 10 glucose. For the apical side, this solution was instead modified by replacing half of NaCl with sodium gluconate and increasing $CaCl_2$ to 2 mM to compensate for calcium buffering caused by gluconate. The basolateral membrane was permeabilized with 250 μg/ml amphotericin B.

For human airway epithelial cells, both apical and basolateral chambers contained 126 mM NaCl, 0.38 mM KH_2PO_4, 2.1 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$, and 10 mM glucose (basolateral membrane was not permeabilized).

During experiments, solutions in both chambers were continuously bubbled with air (FRT cells) or with 5% CO_2 in air (bronchial cells). The hemichambers were connected to DVC-1000 voltage clamps (World Precision Instruments, Inc., Sarasota, FL) via Ag/AgCl electrodes and 1 M KCl agar bridges. Transepithelial currents were digitized using PowerLab 4/25 data acquisition systems and stored on Macintosh computers. All measurements were done at 37°C.

Patch-Clamp Experiments. Recordings of single-channel currents were obtained using the inside-out configuration of the patch-clamp technique. Membrane currents were measured with an EPC-7 (List Medical Instruments, Darmstadt, Germany) patch-clamp amplifier, filtered at 250 Hz, and digitized at 500 Hz using an ITC-16 (InstruTECH Corporation, Port Washington, NY) data translation interface. The pipette solution contained 120 mM CsCl, 10 mM TEA-Cl, 0.5 mM EGTA, 1 mM $MgCl_2$, 40 mM mannitol, and 10 mM cesium-HEPES, pH 7.3. The bath solution contained 160 mM CsCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA (free Ca^{2+} concentration, 176 nM), 10 mM glucose, 10 mM TES, and 1 mM ATP, pH 7.3. CFTR activity was stimulated after excising the patch by the addition of the catalytic subunit of protein kinase A (5 μg/ml; Biaffin GmbH, Kassel, Germany) to the bath solution. Experiments were done at a membrane potential of −60 mV. Recordings of 3 to 10 min were used for the calculation of the P_o.

Data for all experiments are presented as representative traces or as mean ± S.E.M. Statistical analysis was done using the Student's *t* test for unpaired data.

Results

The 2000 compounds included in the Spectrum library were tested on FRT cells expressing ΔF508-CFTR using the YFP-based fluorescence assay. After cell incubation at low temperature to rescue the ΔF508 trafficking defect, each compound was tested at two concentrations (2 and 20 μM) in the presence of maximal forskolin (20 μM). Genistein (50 μM) was included in two wells of each microplate as a positive control. Figure 1A shows a summary of results from the primary screening. A small set of compounds caused an increase of more than 2-fold in the rate of I^- transport with respect to forskolin alone. This increase is comparable with the one caused by genistein (broken line). Most active substances included nicardipine, nimodipine, isradipine, and nitrendipine, all of them DHPs that are used for the treatment of hypertension. Another antihypertensive DHP, nifedipine, was found within the less active compounds (Fig. 1A). Original fluorescence curves for DHPs, along with positive control genistein, forskolin alone, and saline, are shown in Fig. 1B. Other DHPs not included in the library were retrieved from commercial sources or extracted from pharmaceutical preparations and tested with the fluorescence assay. Significant activity was detected for felodipine and niguldipine. On the contrary, amlodipine and lercanidipine were inactive. The full Spectrum library was tested on null FRT cells expressing only the fluorescent protein and not CFTR. No compound was found to stimulate I^- transport on null cells.

All active DHPs were evaluated at various concentrations on FRT cells expressing ΔF508-CFTR cells to generate dose-response relationships (Fig. 1C and Table 1). The most potent compounds were felodipine and nimodipine, with an apparent K_a of 0.7 to 0.8 μM. The rank of potency was the following: nimodipine = felodipine > nitrendipine = isradipine > nifedipine = nicardipine > niguldipine. For comparison, genistein, a known activator of mutant CFTR channels, had a K_a of 23.6 μM. Regarding maximal effect, the effect of most DHPs consisted in a 2.5-fold elevation of the halide transport induced by forskolin alone.

DHPs were tested also on FRT cells expressing wild-type CFTR. Cells were stimulated with a low concentration of forskolin (0.5 μM) with and without compounds at various concentrations. These experiments showed that DHPs are effective also on native CFTR (Fig. 1D). Felodipine was the most potent activator, with a K_a of approximately 0.5 μM. Other compounds of the same family, like nicardipine, were less potent with a K_a closer to that of genistein (4 and 16 μM, respectively). When the same cells were stimulated with maximal forskolin (20 μM), DHPs and genistein did not show further activation (data not shown).

DHPs were tested at different forskolin concentrations on ΔF508-CFTR FRT cells to assess the dependence on CFTR phosphorylation levels. We found that DHPs were effective also with nonmaximal forskolin stimulation, although their potency was proportionally lower. For example, the K_a for nitrendipine was shifted from >50 μM (no forskolin) to 9.8 μM (0.5 μM forskolin) and to ~4.0 μM (2 μM forskolin or greater) (Fig. 2A). We performed also the reverse experiment (i.e., generating forskolin dose-responses in the presence and absence of DHPs). DHPs such as felodipine, nimodipine, nicardipine, nitrendipine, and isradipine significantly enhanced Forskolin effectiveness and potency (Fig. 2B). In particular, the apparent K_a value of forskolin alone was 2.34 ± 0.28 μM (n = 5). In the presence of DHPs, this value was significantly decreased to less than 1 μM (e.g., 0.32 ± 0.01 and 0.57 ± 0.05 for felodipine and nimodipine, respectively; n = 5 for both compounds, $p < 0.001$). DHPs also increased forskolin effectiveness with a more than 2-fold elevation (Fig. 2B). As a control, we tested genistein, which is considered a potentiator acting directly on the CFTR protein (Wang et al., 1998). Genistein increased forskolin potency and effective-

ness similar to DHPs (Fig. 2B). For comparison, we used an indirect CFTR activator, $CFTR_{act}$-16, which works by increasing intracellular cAMP levels, probably through inhibition of phosphodiesterases (Ma et al., 2002b). With this compound, forskolin potency was increased (K_a = 0.17 ± 0.02; n = 5, $p < 0.001$), but its maximal effect was completely unaltered (Fig. 2B).

To evaluate the mechanism of ΔF508-CFTR activation by DHPs, we measured the intracellular cAMP levels. As shown previously (Ma et al., 2002b), the indirect activator $CFTR_{act}$-16 caused a strong elevation of the cAMP levels when combined with a low forskolin concentration (0.5 μM; Fig. 2C). On the contrary, DHPs such as felodipine, nimodipine, and nicardipine were completely ineffective. It is interesting that genistein caused a modest but significant cAMP increase (Fig. 2C).

The ability of DHPs to elicit electrogenic anion transport by ΔF508-CFTR was checked by measuring transepithelial Cl^- currents in FRT cells (Fig. 3). All DHPs identified by the fluorescence assay stimulated Cl^- currents across FRT-ΔF508 epithelia. Felodipine was the most potent agent, with an apparent K_a of approximately 0.4 μM. The maximal effect, as also evidenced by the fluorescence assay, consisted in a more than 2-fold potentiation of the cAMP-activated current. At the end of each experiment, the selective blocker $CFTR_{inh}$-172 (Ma et al., 2002a) was added to demonstrate that currents elicited by DHPs were indeed caused by CFTR activation (Fig. 3).

To elucidate the mechanism leading to CFTR activation, we tested (±)-BayK 8644, a DHP that acts as an activator of voltage-dependent Ca^{2+} channels (Thomas et al., 1985). Despite its opposite effect on Ca^{2+} channels, (±)-BayK-8644 also stimulated ΔF508-CFTR currents similarly to antihypertensive DHPs (Fig. 3). We considered another DHP, MRS-1845, which is a blocker of store-operated Ca^{2+} channels (Harper et al., 2003). MRS-1845 activated ΔF508-CFTR currents at concentrations up to 10 μM, although its effect was smaller than that of the other DHPs. At higher concentrations, MRS 1845 caused instead a slow inactivation (data not shown).

DHPs were also tested as possible activators of the G551D mutant. Cells were stimulated with forskolin at maximal concentration (20 μM) to allow full CFTR phosphorylation and then were treated with increasing concentrations of DHPs. With forskolin alone, no increase of halide transport was detected in G551D cells with the fluorescence assay. The traces were essentially identical with those recorded in the presence of saline solution (Fig. 4A). However, costimulation

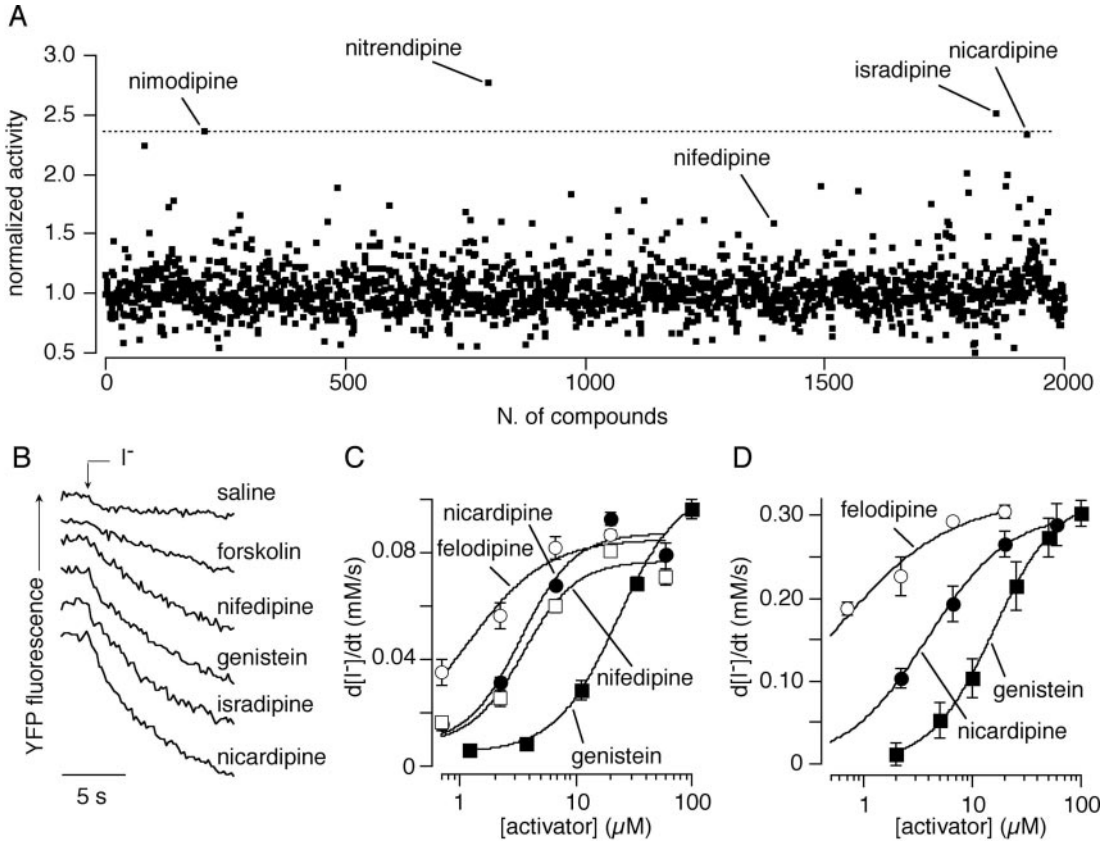


Fig. 1. Identification of antihypertensive DHPs as ΔF508 potentiators. A, summary of activity reported for all 2000 compounds in the Spectrum library. Each dot is the activity elicited by a single compound. FRT cells coexpressing ΔF508-CFTR and the halide-sensitive YFP were first incubated at low temperature to rescue the ΔF508 trafficking defect and then treated with test compounds (20 μM) together with forskolin (20 μM). CFTR activity was determined by measuring the rate of fluorescence quenching induced by extracellular addition of I^-. Data are normalized to the mean activity measured in the presence of forskolin alone. The broken line reports the average activity obtained with positive control genistein (50 μM). Dots corresponding to DHPs are indicated. B, representative fluorescence traces showing the response of ΔF508-CFTR cells to I^- addition under resting conditions (saline) or upon stimulation with forskolin alone or plus activators. C, dose-response relationships obtained for DHPs and genistein from low-temperature rescued ΔF508-CFTR cells. The activity elicited by forskolin (20 μM) alone was subtracted. D, dose-response relationships showing the sensitivity of wild-type CFTR to DHPs and genistein. Cells were costimulated with 0.5 μM forskolin. Data presented in dose-response relationships are the mean ± S.E.M. of 10 and 5 experiments (for ΔF508 and wild-type CFTR, respectively).

with forskolin and DHPs increased halide transport dramatically, as indicated by the steep decrease in cell fluorescence upon the addition of I^-. Dose-response relationships (Fig. 4B and Table 2) revealed that G551D activation required DHP concentrations higher than ΔF508. The potency order for the G551D mutant was the following: felodipine = nicardipine = nitrendipine > nifedipine = nimodipine = isradipine > niguldipine.

In Ussing chamber experiments, very small currents were activated after forskolin stimulation of G551D-CFTR cells. However, the addition of DHPs strongly stimulated mutant CFTR activity (Fig. 4C). Maximal effect for the most effective compounds consisted in 16- to 45-fold increase of the current stimulated by forskolin alone. It is interesting that the maximal response to DHPs like felodipine or isradipine was at least 2-fold larger than that to genistein, a known activator of the G551D mutant (Illek et al., 1999; Zegarra-Moran et al., 2002).

Table 3 shows the structure of tested DHPs, which allows the initial evaluation of the structural requirements for CFTR activation. Various types of substitutions (NO_2, chlorine) of the phenyl ring at *ortho*- and *meta*-positions permit activity. This behavior could resemble that described for DHPs as Ca^{2+}-channel blockers, whereby substituents of the phenyl ring are believed to provide sufficient bulk to lock the ring in particular conformation (Harrold, 2002). On the other hand, substitutions at other positions show differences between CFTR and Ca^{2+} channels. For example, replacement of the ester at R_4 with a NO_2 group produces a Ca^{2+}-channel activator (BayK-8644) whereas the type of effect on CFTR is not changed. Furthermore, bulky groups at R_3 (lercanidipine, niguldipine) or at R_2 (amlodipine), which are optimal for Ca^{2+}-channel blockers, strongly decrease activity on CFTR.

Most of the DHPs used to block voltage-dependent Ca^{2+} channels have a chiral center and show stereoselectivity. Therefore, one of the stereoisomers is usually more potent than the other. We investigated the possibility of stereoselectivity also in the effect of DHPs on CFTR. Niguldipine is commercially available as *S*-(+)- and *R*-(−)-isoforms. In our experiments, the (+)-isomer was slightly more potent than the (−)-form on the ΔF508 mutant (Fig. 5). The *S*-(+)-niguldipine had a modest activity also on G551D-CFTR, whereas the other stereoisomer was completely inactive. We examined also the (+)- and (−)-enantiomers of BayK-8644. Both compounds were effective on ΔF508- and G551D-CFTR, the apparent affinity being lower for the latter mutant (Fig. 5). BayK-8644 showed a modest but significant stereoselectivity for ΔF508 (2-fold difference in K_a; $p < 0.05$) and no stereoselectivity at all for G551D. To further check DHP stereoselectivity, we isolated the (+)- and (−)-stereoisomers from racemic isradipine. Both isradipine enantiomers activated

TABLE 1

Activation properties of 1,4-dihydropyridines measured in FRT cells expressing ΔF508-CFTR

Values for V_{max} and K_a were obtained from experiments of the type shown in Fig. 1C and are the mean ± S.E.M. of 5 to 10 experiments. The activity elicited by forskolin 20 μM alone was subtracted. The compounds are listed in order of decreasing potency. Genistein properties are reported for comparison.

	V_{max}	K_a
	mM/s	*μM*
Racemates		
Nimodipine	0.119 ± 0.002	0.70 ± 0.06
Felodipine	0.095 ± 0.002	0.85 ± 0.15
Nitrendipine	0.112 ± 0.003	2.9 ± 0.3*
Isradipine	0.099 ± 0.003	3.5 ± 0.5
Nifedipine	0.102 ± 0.004	4.9 ± 0.4*
Nicardipine	0.123 ± 0.005	5.9 ± 0.9
Amlodipine	Inactive	
Lercanidipine	Inactive	
Enantiomers		
(+)-BayK-8644	0.106 ± 0.003	2.1 ± 0.2
(+)-Isradipine	0.101 ± 0.003	2.1 ± 0.2
(−)-Isradipine	0.083 ± 0.004	4.1 ± 0.5†
(−)-BayK-8644	0.111 ± 0.005	5.2 ± 0.6†
(+)-Niguldipine	0.080 ± 0.001	30.2 ± 1.1
(−)-Niguldipine	0.053 ± 0.002	45.4 ± 2.0†
Genistein	0.110 ± 0.005	23.6 ± 2.9

* K_a value was significantly ($p < 0.05$) higher than that of the preceding compound.

† K_a value was significantly higher than that of the other isomer.

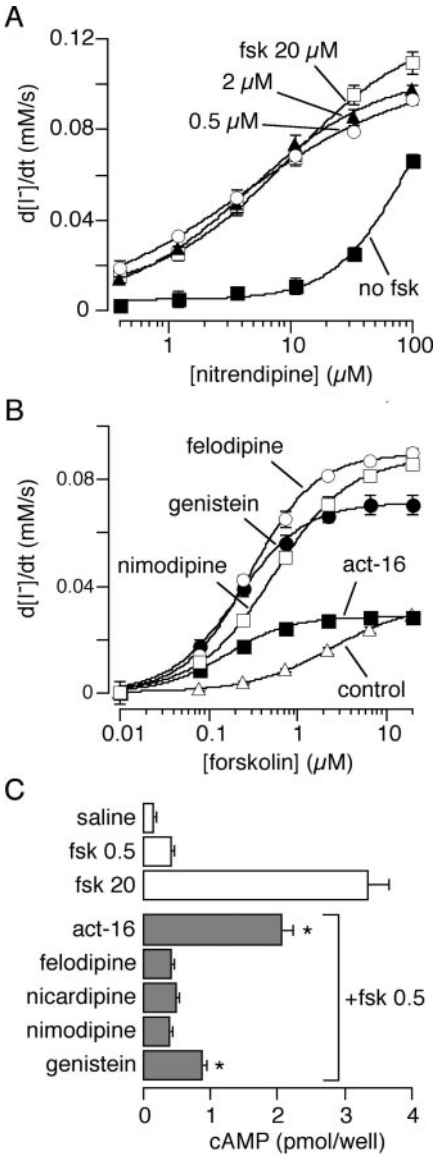


Fig. 2. Properties of ΔF508 activation by DHPs. A, dose-response relationship for nitrendipine at different concentrations of forskolin as indicated. B, dose-response relationship for forskolin with and without 5 μM felodipine, nimodipine, $CFTR_{act}$-16, or 50 μM genistein. C, intracellular cAMP levels measured under resting conditions, with forskolin alone (0.5 or 20 μM), or with forskolin (0.5 μM) plus the indicated CFTR activators. Genistein and $CFTR_{act}$-16 significantly increased cAMP levels ($p < 0.01$) greater than those obtained with forskolin alone. Data reported in the graphs are the mean ± S.E.M. of four to five experiments.

ΔF508-CFTR, with the (+)-form being significantly more potent (Fig. 5). The two different isradipine enantiomers showed different activity on the G551D mutant (Fig. 5).

To further evaluate the mechanism of activation of DHPs on mutant CFTR, we performed patch-clamp experiments. Membrane patches from FRT cells expressing G551D-CFTR were excised in the inside-out configuration. Experimental conditions were set to cancel the possible contribution of voltage-dependent Ca^{2+} channels. First, Ca^{2+} in the pipette and bath solutions was kept at very low levels using the EGTA chelating agent. Second, the membrane potential was clamped at a negative value, which prevents activation of Ca^{2+} channels. Under these conditions, G551D-CFTR channels had a very low activity (P_o = 0.021 ± 0.012; n = 3), despite the presence of the catalytic subunit of the protein kinase A to induce maximal phosphorylation (Fig. 6A). However, subsequent addition of 100 μM felodipine significantly stimulated channel opening (P_o = 0.190 ± 0.033; n = 3; Fig. 6B).

To demonstrate the effect of DHPs on cells expressing CFTR natively, we prepared differentiated primary cultures of airway epithelial cells from patients with CF. Cells were seeded on permeable supports to generate polarized epithelia with high electrical resistance and ion transport properties similar to those of the airway epithelium in vivo (Galietta et al., 1998). In Ussing chamber experiments, the epithelial Na^+ channel current was blocked with apical amiloride 10 μM to allow recording of Cl^- secretion. Nasal epithelial cells from a patient carrying the G551D mutation (Fig. 7A) were largely unresponsive to maximal forskolin (20 μM), with an effect of only 0.05 to 0.2 μA/cm^2, in agreement with previous observations (Zegarra-Moran et al., 2002). However, the application of felodipine (20 μM) elicited a marked increase of the short-circuit current (2.32 ± 0.29 μA/cm^2; n = 3) that was fully blocked by $CFTR_{inh}$-172. Other DHPs were also effective, although to a lower extent (data not shown). Our positive control, genistein, also elicited CFTR-like currents, but the concentrations required were higher (Fig. 7A).

To assess the relevance of functional correction induced by DHPs, we measured CFTR-dependent Cl^- secretion in non-CF airway epithelial cells. After maximal stimulation with forskolin, CFTR currents were 11.4 ± 1.8 μA/cm^2 (n = 8).

We tested also nasal epithelial cells from ΔF508 homozygous patients (Fig. 7B). These cells showed negligible responses to forskolin and to potentiators and only a very small

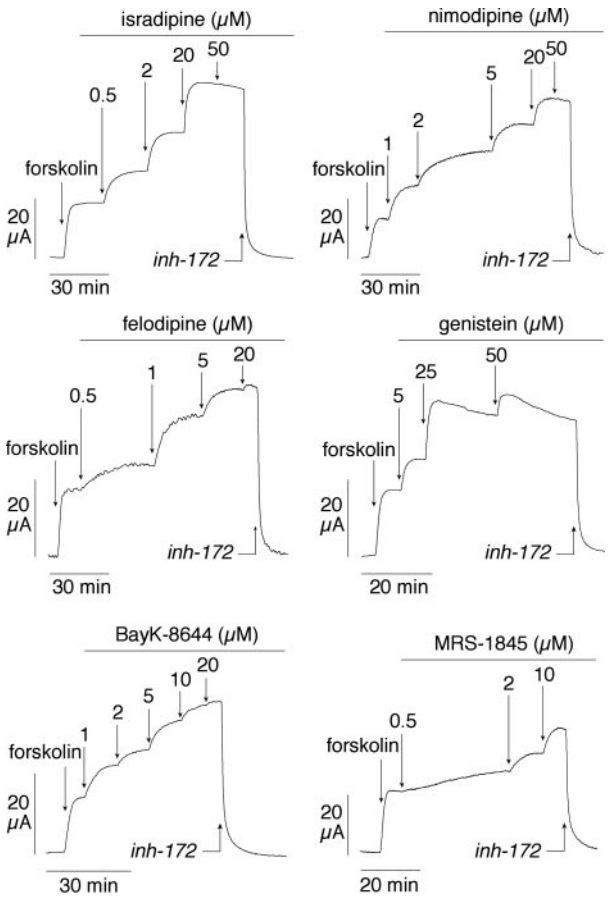


Fig. 3. Activation of ΔF508-CFTR currents by DHPs. Representative traces showing apical membrane Cl^- currents measured in FRT cells expressing ΔF508-CFTR after low-temperature rescue. Similar data were obtained in six to nine experiments for each compound. Cells were stimulated first with forskolin (20 μM) and then with the indicated ΔF508-CFTR activators at various concentrations. CFTR currents were blocked at the end with $CFTR_{inh}$-172 (10 μM).

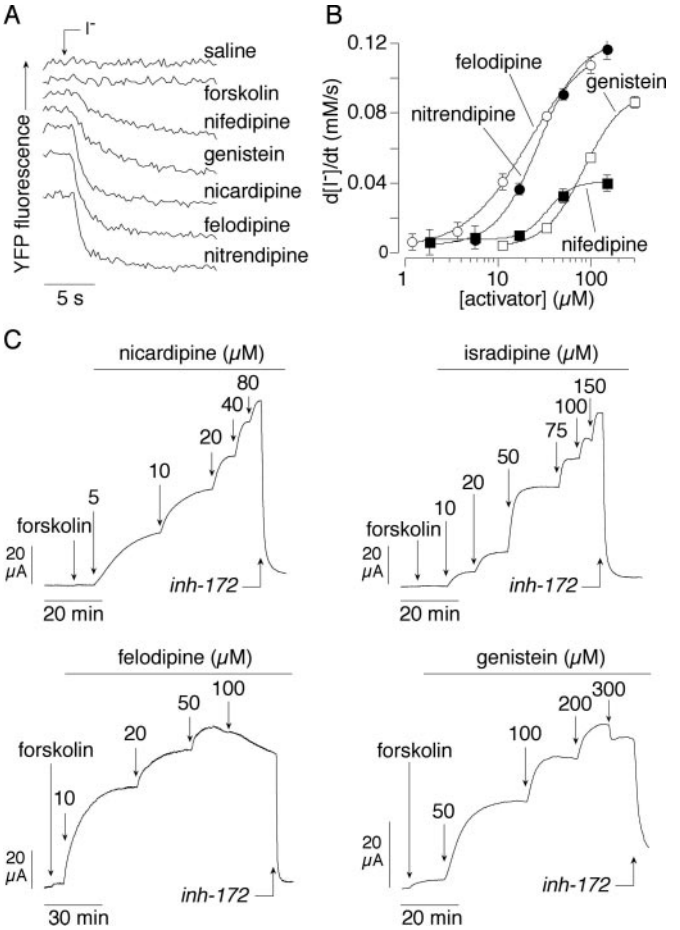


Fig. 4. Activation of G551D-CFTR by DHPs. A, representative traces from microplate reader experiments showing G551D-CFTR activity with saline alone, or forskolin (20 μM) with and without genistein (100 μM) or DHPs (20 μM). B, dose-response relationships obtained with YFP assay for the indicated compounds in the presence of 20 μM forskolin. Data are the mean ± S.E.M. of 10 experiments. C, representative Ussing chamber traces showing G551D-CFTR currents upon stimulation with forskolin (20 μM) and the indicated concentrations of genistein or DHPs. Each recording is representative of 5 to 10 similar experiments.

block by $CFTR_{inh}$-172 (0.2–0.3 $\mu A/cm^2$) when kept at 37°C. Therefore, we incubated the cells at 27°C for 18 to 24 h to allow trafficking of the mutant protein to the plasma membrane. Under these conditions, we observed a small but significant activation of the short-circuit current by forskolin (0.67 ± 0.07 $\mu A/cm^2$; $n = 12$). Many DHPs and genistein were able to further increase the current after forskolin by 100 to 150%, with felodipine being consistently the most effective (Fig. 7B). The total current activated by forskolin plus potentiators was blocked by $CFTR_{inh}$-172 (mean effect, 1.50 ± 0.23 $\mu A/cm^2$; $n = 12$), demonstrating specific induction of CFTR activity.

We tested also felodipine alone, without previous stimulation with forskolin, to assess whether the resting levels of CFTR phosphorylation allow the response to this compound. It is noteworthy that felodipine was largely effective in nonprestimulated conditions with a total activated current comparable with that induced in the presence of forskolin (Fig. 7B).

Discussion

Our screening of a library containing approved drugs has led to the identification of antihypertensive DHPs as a class of molecules able to stimulate the activity of mutant CFTR. Such compounds are widely used to treat essential hypertension (Striessnig et al., 1998; Harrold, 2002). The mechanism of DHPs as antihypertensive agents is derived from the block of L-type, voltage-dependent Ca^{2+} channels. The block is voltage-dependent so that only the channels of smooth muscle cells, which have a relatively depolarized membrane potential, and not those of cardiomyocytes, are mostly affected. Several evidences indicate that the mechanism leading to CFTR activation by DHPs does not involve Ca^{2+}-channel block. First, DHPs stimulate mutant CFTR activity at concentrations significantly higher than those required to inhibit Ca^{2+} currents. Second, CFTR is activated also by racemic BayK-8644, which is instead an activator of L-type Ca^{2+} channels (Thomas et al., 1985; Ravens and Schopper, 1990), and by MRS1845, a DHP which blocks store-operated Ca^{2+} channels (Harper et al., 2003). Third, the effect of DHPs on CFTR is considerably less stereoselective than Ca^{2+}-channel block (Eltze et al., 1990; Handrock and Herzig, 1996; Handrock et al., 1999). As an example, isradipine stereoisomers affect Ca^{2+} channels with a 100-fold difference in potency between the (+) and (−) forms (Handrock et al., 1999). On the contrary, the ratio of (+)- and (−)-isradipine K_a for ΔF508-CFTR is near 2. Furthermore, the two enantiomers of BayK-8644 have opposite effects on voltage-dependent Ca^{2+} channels (Franckowiak et al., 1985; Ravens and Schopper, 1990; Artigas et al., 2003), with the (−)-form being an activator and the (+)-form acting as an inhibitor (although at higher concentrations). On the contrary, both enantiomers activated ΔF508-CFTR currents with comparable potency (only 2.5-fold difference in K_a). In addition, DHPs like lercanidipine and amlodipine, which are potent blockers of Ca^{2+} channels, were ineffective on CFTR. Finally, we performed patch-clamp experiments under conditions that were designed to eliminate the transport operated by Ca^{2+} channels. In such experiments, felodipine was still able to stimulate mutant CFTR.

TABLE 2

Activation properties of 1,4-dihydropyridines measured in FRT cells expressing G551D-CFTR

Values for V_{max} and K_a were obtained from experiments of the type shown in Fig. 4B and are the mean ± S.E.M. of 5 to 10 experiments. The activity elicited by forskolin 20 μM alone was negligible and therefore not subtracted. The compounds are listed in order of decreasing potency. Genistein properties are reported for comparison.

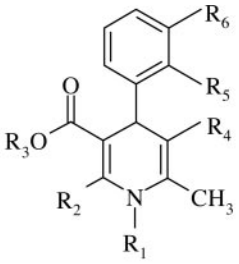
	V_{max}	K_a
	mM/s	*μM*
Racemates		
Felodipine	0.145 ± 0.013	23.3 ± 2.1
Nicardipine	0.105 ± 0.006	25.9 ± 2.1
Nitrendipine	0.130 ± 0.004	26.3 ± 1.0
Nifedipine	0.058 ± 0.005	34.2 ± 1.9*
Nimodipine	0.078 ± 0.004	36.2 ± 2.3
Isradipine	0.100 ± 0.006	37.5 ± 2.3
Amlodipine	Inactive	
Lercanidipine	Inactive	
Enantiomers		
(+)-Isradipine	0.085 ± 0.006	25.8 ± 2.2
(−)-BayK-8644	0.081 ± 0.002	30.6 ± 1.4
(+)-BayK-8644	0.116 ± 0.005	36.5 ± 3.1
(+)-Niguldipine	0.034 ± 0.002	87 ± 4
(−)-Isradipine	0.049 ± 0.003	88 ± 5†
(−)-Niguldipine	Inactive	
Genistein	0.097 ± 0.007	90 ± 10

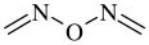
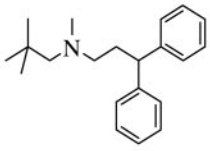
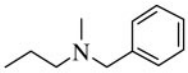
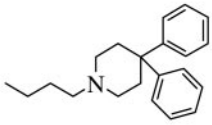
* K_a value was significantly ($p < 0.05$) higher than that of the preceding compound.
† K_a value was significantly higher than that of the other isomer.

The mechanism underlying the activation of Cl^- transport by DHP remains unclear but probably involves direct interaction with CFTR protein. An evidence in favor of this conclusion is represented by the differences of DHP potency for ΔF508 and G551D mutants. For ΔF508-CFTR, felodipine was significantly more potent than nitrendipine and nicardipine. Conversely, for G551D, the K_a values of these three DHPs were comparable and significantly higher than that for ΔF508. The different sensitivity of the two CFTR mutants, which differ by single amino acid alterations at NBD1, strongly suggests that the DHP binding site lies in the CFTR protein itself. An alternative indirect mechanism, involving the inhibition of phosphodiesterases and consequent elevation of cAMP, seems unlikely. In fact, DHPs activate CFTR even in the presence of a high forskolin concentration, when cAMP is maximally elevated, and are unable to increase cAMP by themselves. Conversely, indirect CFTR activators, like $CFTR_{act}$-16, a strong cAMP-elevating agonist (Ma et al., 2002b), are not able to potentiate ΔF508-CFTR activity in the presence of maximal forskolin. The different behavior between putative direct CFTR openers and cAMP-elevating agents is also demonstrated by experiments such as those shown in Fig. 2B. The forskolin dose-response is shifted by $CFTR_{act}$-16 to lower forskolin concentrations without increasing the maximal effect. On the contrary, DHPs increase both the maximal effect and the potency of forskolin. This behavior is similar to that of genistein, which is considered to be a direct CFTR channel activator (Wang et al., 1998). In contrast to DHP, genistein has also a modest ability to increase intracellular cAMP. This may be caused by a weak activity of genistein as a phosphodiesterase inhibitor (Burvall et al., 2002).

The decreased channel activity of ΔF508 and G551D mutants is considered to be the result of an intrinsic defect in CFTR protein function. Therefore, small molecules like DHPs may interact with important CFTR domains, like NBD-1, to favor conformational changes that lead to channel opening and Cl^- transport. To this respect, it is interesting to

TABLE 3
Structures of tested 1,4-dihydropyridines



	R_1	R_2	R_3	R_4	R_5	R_6
Amlodipine	H	$CH_2O(CH_2)_2NH_2$	CH_2CH_3	$COOCH_3$	Cl	H
BayK-8644	H	CH_3	CH_3	NO_2	CF_3	H
Felodipine	H	CH_3	CH_2CH_3	$COOCH_3$	Cl	Cl
Isradipine	H	CH_3	CH_3CHCH_3	$COOCH_3$		
Lercanidipine	H	CH_3		$COOCH_3$	H	NO_2
MRS 1845		CH_3	CH_2CH_3	$COOCH_3$	H	NO_2
Nicardipine	H	CH_3		$COOCH_3$	H	NO_2
Nifedipine	H	CH_3	CH_3	$COOCH_3$	NO_2	H
Niguldipine	H	CH_3		$COOCH_3$	H	NO_2
Nimodipine	H	CH_3	CH_3CHCH_3	$COO(CH_2)_2OCH_3$	H	NO_2
Nitrendipine	H	CH_3	CH_2CH_3	$COOCH_3$	H	NO_2

note that DHPs have been found to modulate the activity of P-glycoprotein, another member of the ATP-binding cassette protein superfamily (Ferry et al., 1992; Hollt et al., 1992; Takara et al., 2002). This effect is believed to be independent of Ca^{2+}-channel block and rather is caused by direct interaction with the P-glycoprotein. Photoaffinity labeling has been used to investigate the DHP binding site (Borchers et al., 2002). After covalent binding and proteolytic digestion, mass spectrometry identified the labeled fragment, which seemed to include one of the NBDs.

In a previous screening involving 100,000 random compounds, six classes of ΔF508 potentiators were found, including a novel DHP, labeled as $\Delta F508_{act}$-05 (Yang et al., 2003), different from the ones used for antihypertensive therapy. Subsequent analysis of $\Delta F508_{act}$-05 analogs did not reveal other active DHPs (Yang et al., 2003). Our results show now that antihypertensive DHPs are an interesting class of CFTR openers that could be exploited to develop a strategy for the pharmacological correction of the CF defect. Approved drugs have an obvious advantage over novel chemical entities iden-

tified by HTS of random compound libraries. In particular, for DHPs, a large amount of information is available on pharmacokinetics and toxicity (Harrold, 2002). Consequently, clinical trials could be initiated more rapidly. However, we need to consider that the DHP effect on mutant CFTR requires concentrations higher than those needed for antihypertensive activity. Various strategies could be envisioned to solve this problem. First, it could be possible to exploit the different stereoselectivity shown by DHPs for voltage-dependent Ca^{2+} channels and CFTR. The block of Ca^{2+} channels is highly stereoselective, with one of the stereoisomers having much higher potency than the other. Conversely, CFTR shows comparable sensitivity to DHP enantiomers. Therefore, one possible approach is to use the stereoisomer that is less potent on Ca^{2+} channels as CFTR potentiator. Second, a pharmaceutical preparation could be developed for aerosol administration of a selected DHP. In this way, effective concentrations could be reached locally in the CFTR-expressing airway epithelium with minimal systemic effect. Finally, it has to be considered that other clinically approved DHPs, not available for our study, may have a higher affinity for CFTR.

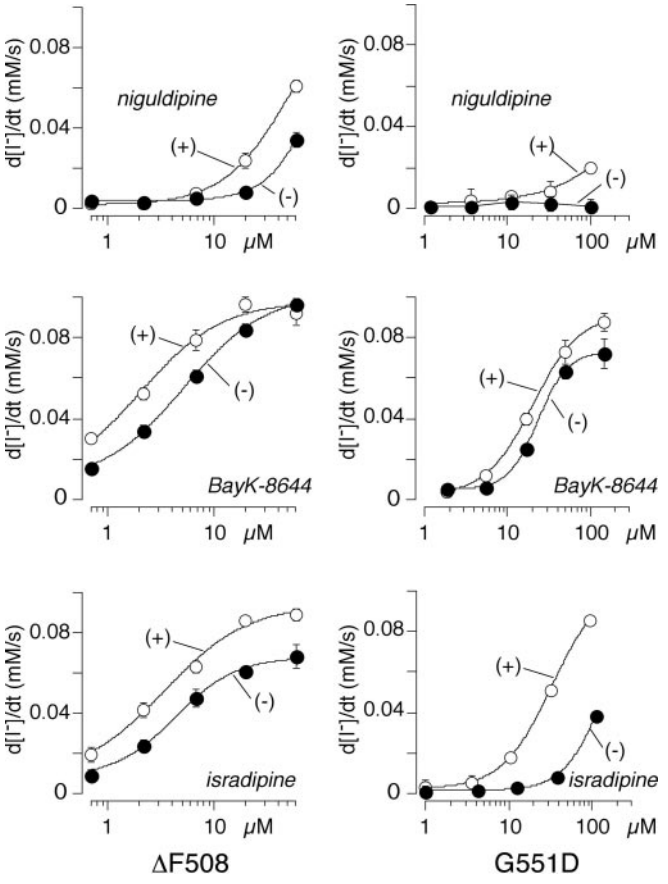


Fig. 5. Activity of DHP enantiomers. Dose-response curves for niguldipine, BayK-8644, and isradipine enantiomers. Data (mean ± S.E.M. of 5–10 experiments) were obtained from ΔF508 (left) and G551D (right) using the YFP assay. Forskolin (20 μM) was present in all conditions.

Our experiments on human airway epithelial cells demonstrate that DHPs are effective also on a native cell system expressing endogenous CFTR. The effectiveness clearly depends on the type of CFTR mutation. For ΔF508, the use of a potentiator alone is probably of little help because most of the protein is trapped inside the cell. However, CFTR activators, such as DHPs, could be combined with other treatments that improve the trafficking of the mutant CFTR to the plasma membrane. In our experiments on ΔF508 nasal epithelial cells corrected at low temperature, the effect of DHPs and genistein consisted in a 100 to 150% stimulation with respect to forskolin alone, and the total current activated by forskolin plus potentiators was 10 to 13% of the CFTR current measured in non-CF airway cells. This is of therapeutic relevance, given that only 5 to 10% of normal CFTR function seems to be required to prevent or slow down lung pathology in patients with CF. It has to be noted that the response to potentiators in airway epithelial cells was relatively smaller than that measured in FRT cells (100–150% versus 250%). This may imply different levels of phosphorylation (and therefore activity) attained by stimulation with forskolin alone. Despite this lower efficacy, the effect of potentiators is still significant because it causes at least a doubling of the current activated by forskolin alone. Therefore, compounds like DHPs could potentiate the benefits of treatment with small molecules able to correct the ΔF508 trafficking defect.

For CF mutations other than ΔF508, potent and selective CFTR activators could be very important to developing a single drug therapy. One classic example is G551D, which is a class III mutation characterized by a severe channel-open-

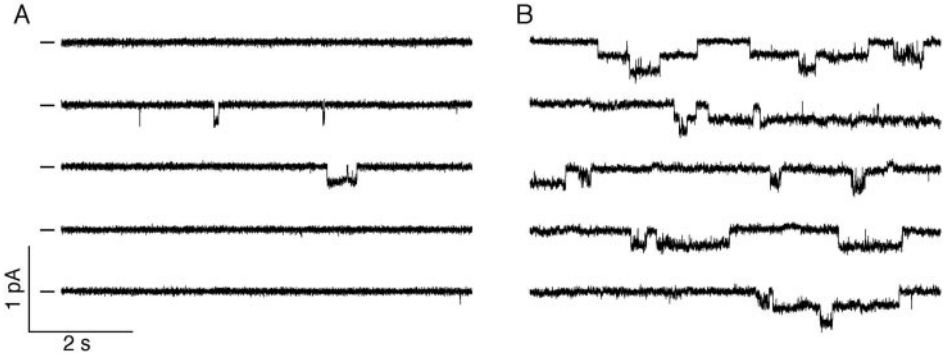


Fig. 6. Activation of mutant CFTR by felodipine in excised membrane patches. A, traces showing continuous recordings of currents from and inside-out membrane patch excised from a FRT cell expressing G551D-CFTR. The membrane voltage was clamped at −60 mV (negative on the cytosolic side). The bath solution contained the catalytic subunit of protein kinase A to induce channel phosphorylation. The closed channel level is shown as small lines to the left of each trace. Rare channel openings (downward deflections) were detected under these conditions. B, traces from the same experiment showing activation of G551D channels by the addition of 100 μM felodipine to the bath solution. The traces are representative of three similar experiments.

ing deficit (Gregory et al., 1991). We have shown previously that genistein is an effective activator of the G551D mutant, with a level of functional correction close to 20% in FRT and nasal epithelial cells (Zegarra-Moran et al., 2002). Our present results show that the best DHP, felodipine, also stimulated Cl^- secretion in G551D airway epithelial cells up to a level close to 20% of that measured in non-CF cells. They are therefore very promising as drugs for class III mutations, although the potency has to be largely improved.

DHPs are also an interesting class of small molecules as basic research tools to investigate CFTR structure/function. The different sensitivity of ΔF508 and G551D mutants suggests that the DHP binding site resides in the NBDs. Radiolabeled and photoactivable DHPs are already available commercially or synthesized by various research groups (Hockerman et al., 1997; Borchers et al., 2002). They have been used to investigate the DHP interaction with P-glycoprotein. Similar studies could be carried out on CFTR to identify the binding site(s) and the possible interaction with other CFTR activators or inhibitors.

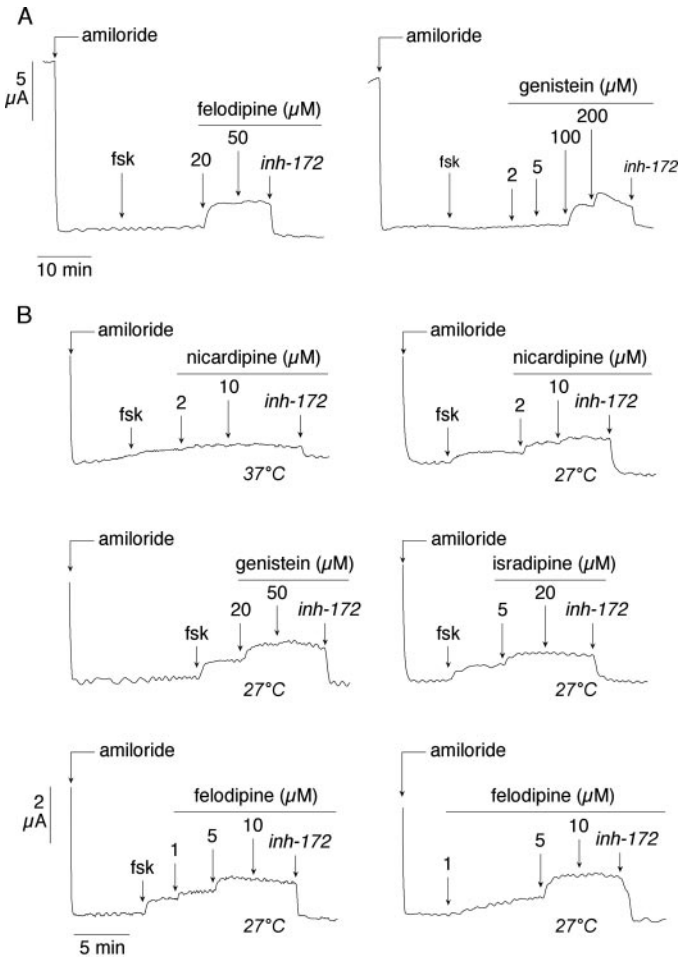


Fig. 7. Stimulation of CFTR currents in human airway epithelial cells. A, representative experiments on nasal epithelial cells from a patient with CF carrying the G551D mutation. The traces depict the short-circuit current changes upon the addition of amiloride (10 μM), symmetrical forskolin (fsk; 20 μM), and apical felodipine or genistein at the indicated concentrations. The blocker $CFTR_{inh}$-172 (10 μM) was added at the end of each experiment. B, experiments from nasal epithelial cells from a ΔF508 homozygote patient with CF. As indicated, cells were kept at 37°C or incubated at 27°C for 18 to 24 h before the experiments. During short-circuit current experiments, cells were sequentially treated with amiloride (10 μM), forskolin (20 μM), genistein, or DHPs at the indicated concentrations, and $CFTR_{inh}$-172 (10 μM). In one set of experiments, felodipine was applied without previous forskolin stimulation (lower right trace). Each trace in the figure is representative of three to four similar experiments.

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