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The relationship between cell proliferation, Cl^- secretion, and renal cyst growth: A study using CFTR inhibitors

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The relationship between cell proliferation, Cl^- secretion, and renal cyst growth: A study using CFTR inhibitors.

Background. In autosomal-dominant polycystic kidney disease (ADPKD), cAMP-stimulated cell proliferation and Cl^- secretion via the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel drive the enlargement of fluid-filled epithelial cysts. To investigate how CFTR blockers inhibit cyst growth, we studied cAMP-dependent Cl^- secretion, cell proliferation, and cyst growth using type I Madin Darby canine kidney (MDCK) cells as a model of renal cyst development and growth.

Methods. We grew MDCK cysts in collagen gels in the presence of the cAMP agonist forskolin, measured Cl^- secretion with the Ussing chamber technique, and assayed cell proliferation using nonpolarized and polarized MDCK cells. To inhibit CFTR, we used glibenclamide, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), genistein, and the specific CFTR inhibitor $CFTR_{inh}$-172. As controls, we tested the effects of blockers of other types of apical membrane Cl^- channels and inhibitors of basolateral membrane ion channels and transporters.

Results. In the absence of inhibitors of transepithelial ion transport, forskolin stimulated dramatic cyst growth. CFTR blockers and inhibitors of basolateral membrane ion channels and transporters retarded cyst growth. In contrast, blockers of other types of apical membrane Cl^- channels, which were without effect on CFTR, failed to inhibit cyst growth. Inhibition of cyst growth by CFTR blockers was correlated with inhibition of cAMP-stimulated Cl^- current (correlation coefficient = 0.81; $P < 0.05$), but not cell proliferation (correlation coefficient = 0.50; $P > 0.05$).

Conclusion. Our data suggest that CFTR blockers might retard cyst growth predominantly by inhibiting fluid accumulation within the cyst lumen.

Autosomal-dominant polycystic kidney disease (ADPKD) is one of the most prevalent single gene disorders to affect humans, with an incidence of approximately 1 in 1000 live births in all ethnic groups [1]. The disease is caused by mutations in the polycystin proteins that initiate a cascade of events that result in the formation of multiple fluid-filled epithelial cysts [2, 3]. The insidious enlargement of cysts progressively destroys the architecture of the kidney, leading to severe renal failure. At present, there are no cures for ADPKD, which accounts for approximately 8% to 10% of patients requiring kidney transplantation and dialysis [1].

The development and growth of ADPKD cysts involve the proliferation of immature epithelial cells, changes in the extracellular matrix, and the accumulation of fluid within the cyst cavity [2, 3]. The growth of ADPKD cysts is accelerated by activation of the cAMP signal-transduction cascade, with elevation of the intracellular concentration of cAMP stimulating the proliferation of ADPKD epithelial cells [4, 5] and fluid accumulation within the cyst cavity [3, 6]. The accumulation of fluid within ADPKD cysts is powered by transepithelial Cl^- movements that resemble cAMP-stimulated Cl^- secretion by secretory epithelia, such as those that line the respiratory airways, pancreatic ducts, and small intestine [3, 7]. In secretory epithelia, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel located in the apical membrane provides a pathway for Cl^- exit from the cell, and controls the rate of transepithelial fluid and electrolyte transport [7]. Malfunction of CFTR causes the genetic disease cystic fibrosis (CF) [7].

Several lines of evidence suggest that the CFTR Cl^- channel plays a key role in the accumulation of fluid and electrolytes within the lumen of ADPKD cysts. First, immunocytochemistry studies demonstrated that CFTR is located in the apical membrane of ADPKD cysts [8, 9]. Second, Cl^- currents with properties and regulation identical to those of CFTR have been identified in ADPKD epithelial cells using the whole-cell patch-clamp technique [9]. Third, CFTR antisense oligonucleotides inhibited cAMP-stimulated fluid secretion by ADPKD epithelia [10]. Taken together, these data suggest that

Key words: CFTR, ADPKD, Cl^- secretion, cell proliferation, Cl^- channel, Cl^- channel blockers.

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Cl^- transport by CFTR drives the accumulation of fluid within the lumen of ADPKD cysts. They also suggest strongly that inhibitors of the CFTR Cl^- channel might retard cyst growth by blocking fluid accumulation within the cyst lumen.

However, other lines of evidence suggest that inhibitors of the CFTR Cl^- channel might diminish cyst growth by retarding cell proliferation. First, blockers of a variety of ion channels, including the volume-regulated anion channel (VRAC), inhibit cell division [11, 12]. Second, in addition to its best-characterized role as a regulated Cl^- channel, CFTR modulates the activity of other ion channels and transporters in epithelial cells, including VRACs and volume-sensitive K^+ channels [7, 13, 14]. Third, expression of wild-type CFTR in CF lymphocytes restored the cell-cycle-dependence of Cl^- permeability in these cells [15]. These data raise the possibility that inhibitors of the CFTR Cl^- channel might retard cyst growth by inhibiting cell proliferation.

Thus, the aim of this study was to test the effects of inhibitors of the CFTR Cl^- channel on cyst growth and identify their mechanism of action. For this work, we used type I Madin Darby canine kidney (MDCK) epithelial cells, a valuable model system to investigate renal cyst development and growth [3]. These cells endogenously express CFTR, form polarized epithelia on permeable filter supports, and cysts in collagen gels [16, 17]. To inhibit CFTR, we used the sulfonylurea drug glibenclamide, the arylaminobenzoate 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), the flavonoid genistein, the thiazolidinone $CFTR_{inh}$-172, and H-89, a membrane-permeant selective inhibitor of protein kinase A (PKA). Glibenclamide and NPPB are open-channel blockers of the CFTR Cl^- channel that inhibit Cl^- flow through the CFTR pore [18, 19]. Genistein is widely used to potentiate CFTR Cl^- currents (for review, see [20]). However, elevated concentrations of the drug inhibit CFTR by disrupting channel gating [21]. $CFTR_{inh}$-172 is a potent, specific inhibitor of the CFTR Cl^- channel that was identified by high-throughput screening [22]. Like genistein [21], $CFTR_{inh}$-172 inhibits CFTR by altering channel gating [23]. H-89 inhibits CFTR by impeding PKA-dependent phosphorylation, a prerequisite for channel opening [7, 24]. As controls, we tested the effects on cyst growth of inhibitors of other types of apical membrane Cl^- channels and blockers of basolateral membrane ion channels and transporters. In this way, we could establish the importance of different types of ion channels and transporters for cyst growth.

METHODS

Cells and cell culture

Type I MDCK epithelial cells [16] were a generous gift from Professor M.J. Welsh (University of Iowa, Iowa City, IA, USA). Cells were cultured in MDCK media (a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (all from Invitrogen, Ltd., Paisley, UK) at 37°C in a humidified atmosphere of 5% CO_2.

Cyst growth

To grow cysts, MDCK cells were cultured in collagen gels in the presence of the cAMP agonist forskolin using a modification of the method of Grantham et al [17]. Individual wells of a 24-well plate containing 0.4 mL of ice-cold Vitrogen (~3.0 mg mL^{-1} collagen; Cohesion Technologies, Inc., Palto Alto, CA, USA) supplemented with 10% (v/v) 10× minimum essential medium, 10 mmol/L HEPES, 27 mmol/L $NaHCO_3$, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (pH 7.4 with NaOH) were seeded with ~800 MDCK cells. After gelation of the Vitrogen, 1.5 mL of MDCK media containing forskolin (10 μmol/L) was added to each well of the 24-well plate. Plates were maintained at 37°C in a humidified atmosphere of 5% CO_2, and the MDCK media containing forskolin changed every two days.

Six days after seeding collagen gels with MDCK cells, cysts were readily detected at ×100 magnification using an inverted microscope with phase contrast optics (Leica, model DMIL, Milton Keynes, UK). To test the effect of drugs on cyst growth, drugs were added to MDCK media in the continued presence of forskolin on day six. MDCK media containing forskolin and drugs was changed every two days for a total period of six days. Photographs of individual cysts were taken before the addition of drugs and at two-day intervals over the duration of the experiment. To identify individual cysts, each cyst was assigned a unique reference number using a grid placed below the 24-well plate.

In some experiments, we tested the effects of $CFTR_{inh}$-172 on cyst formation by adding the drug to MDCK media in the continued presence of forskolin from day 0 onward. At day six we compared the number and volume of cysts grown in the absence and presence of $CFTR_{inh}$-172.

Cyst number and volume measurements

To determine cyst numbers, we counted all cysts in each well that had a diameter larger than 50 μm on day six. To calculate cyst volumes, the diameter of cysts was measured directly from photographs of cysts using images that had been magnified by identical amounts. By assuming that cysts are spherical in shape, we calculated cyst volume ($4/3 \times \pi \times r^3$).

Ussing chamber experiments

To grow MDCK cells as polarized epithelia, cells were seeded directly onto permeable filter supports

(Millicell-PCF culture plate inserts, 0.4 μm pore size, 12 mm diameter, Millipore Corp.; Fisher Scientific UK, Loughborough, UK) at a density of 3×10^5 cells per 0.6 cm^2. Every second day after seeding, we changed the MDCK media and measured transepithelial resistance (R_t) using an epithelial voltohmmeter (EVOM; World Precision Instruments, Stevenage, UK). On day eight (when $R_t = 2.2 \pm 0.2$ kΩ; mean ± SEM; $N = 40$), we removed the media from the apical side of the epithelium to form an air-liquid interface because this maneuver enhances the expression of CFTR at the apical membrane of MDCK epithelia [16]. On day 10 (when $R_t = 5.0 \pm 0.3$ kΩ; $N = 40$), we used MDCK epithelia for experiments.

To test the effects of drugs on Cl^- channels located in the apical membrane, we permeabilized the basolateral membrane with nystatin, clamped transepithelial voltage at 0 mV, and imposed a large Cl^- concentration gradient across the epithelium. Under these conditions, we measured apical Cl^- current ($I_{Cl}{}^{apical}$) using a Warner Instrument Corp. Epithelial Voltage-Clamp (model EC-825; Harvard Apparatus, Ltd., Edenbridge, UK) and AxoScope data acquisition and analysis software (version 8.2, Axon Instruments, Inc., Union City, CA, USA). MDCK epithelia were mounted in modified Ussing chambers (Warner Instrument Corp., Dual Channel Chamber, model U-2500; Harvard Apparatus Ltd.). The basolateral membrane of MDCK epithelia was bathed in a solution containing (mmol/L): 140 NaCl, 5 KCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, and 4.2 $NaHCO_3$, pH 7.2 with Tris ([Cl^-], 149 mmol/L); osmolarity, 292 ± 0.4 mOsm ($N = 9$). The composition of the solution bathing the apical membrane of MDCK epithelia was identical to that of the basolateral solution, with the exception that 133.3 mmol/L Na gluconate + 2.5 mmol/L NaCl and 5 mmol/L K gluconate replaced 140 mmol/L NaCl and 5 mmol/L KCl, respectively, to give a Cl^- concentration of 14.8 mmol/L (osmolarity, 282 ± 0.3 mOsm; $N = 9$). To compensate for the Ca^{2+} buffering capacity of gluconate, we increased the concentration of Ca^{2+} to 5.7 mmol/L in the apical solution; all solutions were maintained at 37°C and bubbled with O_2.

To permeabilize the basolateral membrane to monovalent ions, we added nystatin (0.36 mg/mL) to the basolateral solution. The effectiveness of nystatin permeabilization was assessed as previously described [25]. We clamped transepithelial voltage (referenced to the basolateral solution) at 0 mV, and recorded $I_{Cl}{}^{apical}$ continuously using a Digidata 1200 interface (Axon Instruments, Inc.) and AxoScope software at a sampling rate of 0.5 kHz. To measure R_t, we applied 5 mV voltage steps (duration 0.1 seconds, period 10 seconds), and then calculated R_t using Ohm's law. The resistance of the filter and solutions, in the absence of cells, was subtracted from all measurements. Flow of current from the basolateral to the apical solution is shown as an upward deflection.

To test the effects of drugs on basolateral membrane ion channels and transporters, we measured short-circuit current (I_{sc}) as described for $I_{Cl}{}^{apical}$, with the exception that the basolateral membrane was intact. During prolonged recordings of $I_{Cl}{}^{apical}$ or I_{sc}, rundown of cAMP-stimulated Cl^- current was observed. For example, 2500 seconds after stimulation by forskolin (10 μmol/L), $I_{Cl}{}^{apical}$ declined by 19% ± 6% ($N = 6$). To compensate for current rundown when quantifying drug inhibition, we subtracted from the drug-induced current inhibition an amount of current equivalent to that of the rundown observed at the specific time point used to determine current inhibition.

Cell proliferation assays

Nonpolarized cells. To test the effect of drugs on the proliferation of MDCK cells, we used cells grown in MDCK media containing either 0.01% or 10% FBS. For these experiments, we used MDCK cells grown in media containing 5% FBS and 1% insulin-transferrin-selenium-X supplement (ITS-X; Invitrogen, Ltd.). On day −1, 3.5×10^4 MDCK cells were seeded in individual wells of a 12-well plate containing MDCK media with 1% FBS and 1% ITS-X supplement. On day 0, the concentration of FBS was either reduced further to 0.01%, or increased to 10% and forskolin (10 μmol/L) and drugs were added to the MDCK media. MDCK media containing forskolin and drugs was changed every two days for a total period of six days. To determine the number of cells per well, MDCK cells were harvested using trypsin (0.25% wt/vol), centrifuged at 1200 rpm for 5 minutes, and resuspended in 1 mL of MDCK media before counting using a hemocytometer. The viability of MDCK cells was determined by staining with trypan blue (0.2%).

Polarized cells. For cell proliferation assays using polarized MDCK epithelia, we seeded cells directly onto transparent permeable filter supports (Transwell-clear insert, 0.4 μm pore size, 12 mm diameter, Corning, Ltd., High Wycombe, UK) at a density of 3×10^5 cells per 1.1 cm^2 using media with 5% FBS, 1% ITS-X supplement, and forskolin (10 μmol/L). Every day, we measured R_t using an EVOM, and on every second day, we changed the MDCK media. On day five (when $R_t = 1.1 \pm 0.1$ kΩ; $N = 27$), we reduced the concentration of FBS to 1%. On day six, we further reduced the FBS concentration to 0.01% and added drugs to the media. After measuring R_t, we removed the media from the apical side of the epithelium to form an air-liquid interface for the reasons described above. MDCK media containing forskolin and drugs were changed every two days for a total period of six days. To determine the number of cells per filter, cells were harvested and counted, as described above.

Lactate dehydrogenase assay. For cytotoxicity studies, we used a nonradioactive cytotoxicity detection kit that measures the activity of lactate dehydrogenase (LDH) released from cells (Roche Products, Ltd., Welwyn Garden City, UK). We grew MDCK cells as described above for cell proliferation studies of nonpolarized MDCK cells, with the exception that cells were grown in 96-well plates (Corning, Ltd.) using a seeding density of 8×10^2 cells per well. Cells were treated with drugs for 24 hours in the presence of 0.01% FBS and 1% ITS-X supplement before performing the LDH assay according to the manufacturer's instructions.

Analysis of the relationships between cyst growth, Cl^- secretion, and cell proliferation

To compare the potency of the drugs on cyst growth, Cl^- secretion, and cell proliferation, we designated values in the presence of forskolin (10 μmol/L), but absence of drugs, as 100%. We then expressed values in the presence of drugs as a percentage of those in the presence of forskolin alone. To investigate correlations between different groups of data, we used Pearson's correlation test.

Reagents

Barium, bumetanide, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), forskolin, genistein, glibenclamide, HEPES, niflumic acid, nystatin, ouabain, tamoxifen, and tetraethylammonium (TEA) chloride were purchased from the Sigma-Aldrich Company, Ltd. (Poole, UK). NPPB was obtained from Semat Technical (UK) Ltd. (St. Albans, UK), and H-89 (N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl; Calbiochem) was purchased from Merck Biosciences, Ltd. (Nottingham, UK). Calix[4]arene was a generous gift of Professor R.J. Bridges and Dr. A.K. Singh (University of Pittsburgh, Pittsburgh, PA, USA). $CFTR_{inh}$-172 was a generous gift of Professor A.S. Verkman (University of California, San Francisco, CA, USA). All other chemicals were of reagent grade.

Forskolin was dissolved in methanol, tamoxifen in ethanol and barium, calix[4]arene and TEA in distilled water; all other drugs were dissolved in DMSO. Stock solutions were stored at −20°C and diluted in either MDCK media or physiologic salt solutions to achieve final concentrations immediately before use. Precautions against light-sensitive reactions were observed when using bumetanide, genistein, nystatin, ouabain, tamoxifen, and TEA. DMSO (0.4% v/v) and ethanol (0.2% v/v) were without effect on cyst growth, cell proliferation, and I_{Cl}^{apical} (data not shown).

Statistics

Results are expressed as mean ± SEM of N observations. To test for differences between groups of data, we used Student t test. Differences were considered statistically significant when $P < 0.05$. Tests were performed using SigmaStat™ (version 2.03, Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

Inhibitors of transepithelial ion transport diminish cyst growth

To test the effects of inhibitors of the CFTR Cl^- channel on cyst growth, we used type I MDCK cells that form cysts when grown in collagen gels in the presence of cAMP agonists [17]. Following the seeding of collagen gels with MDCK cells and incubation in media containing forskolin (10 μmol/L), we first observed cysts after 2 to 3 days. Cysts progressively increased in size and, after six days, they were readily detected at ×100 magnification (Fig. 1A). Figure 1A and B demonstrates that in the sustained presence of forskolin (10 μmol/L), cysts continued to enlarge over the following six days. During this period, cyst volume increased by 687 ± 50% (day 6, 0.0019 ± 0.0003; day 12, 0.0094 ± 0.0012 mm^3; $N = 186$; $P < 0.01$; Student paired t test; Fig. 2). Based on these data, we examined the effects of inhibitors of the CFTR Cl^- channel on cyst growth between day 6 and 12 after seeding collagen gels with MDCK cells.

To inhibit the CFTR Cl^- channel, we used $CFTR_{inh}$-172 (10 μmol/L), genistein (100 μmol/L), glibenclamide (100 μmol/L), H-89 (10 μmol/L), and NPPB (50 μmol/L). Glibenclamide and NPPB are open-channel blockers of CFTR that inhibit Cl^- flow through the channel, $CFTR_{inh}$-172 and genistein are allosteric blockers of CFTR that disrupt channel gating, and H-89 is an inhibitor of PKA that phosphorylates CFTR prior to channel opening [7, 18, 19, 21, 23, 24]. As controls, we tested the effects on cyst growth of drugs that inhibit other types of apical membrane Cl^- channels and blockers of ion channels and transporters that are located in the basolateral membrane of epithelia [7, 26]. To inhibit other types of apical membrane Cl^- channels, we used calix[4]arene (10 μmol/L), DIDS (200 μmol/L), niflumic acid (100 μmol/L) and tamoxifen (10 μmol/L). DIDS inhibits other types of epithelial Cl^- channels, but is without effect on CFTR when added to the outside of cells [26]. Niflumic acid inhibits Ca^{2+}-activated Cl^- channels, tamoxifen inhibits VRACs, and calix[4]arene inhibits outwardly rectifying Cl^- channels [27] [abstract; Singh AK et al, *Pediatr Pulmonol* 13(Suppl):237, 1996]. At the concentrations used, calix[4]arene is without effect on CFTR [abstract; Singh AK et al, *Pediatr Pulmonol* 13(Suppl):237, 1996], while tamoxifen and niflumic acid inhibit CFTR activity by 10% and 25%, respectively [28, 29]. To inhibit ion channels and transporters in the basolateral membrane, we used barium (1 mmol/L), bumetanide (100 μmol/L), ouabain (1 μmol/L), and TEA

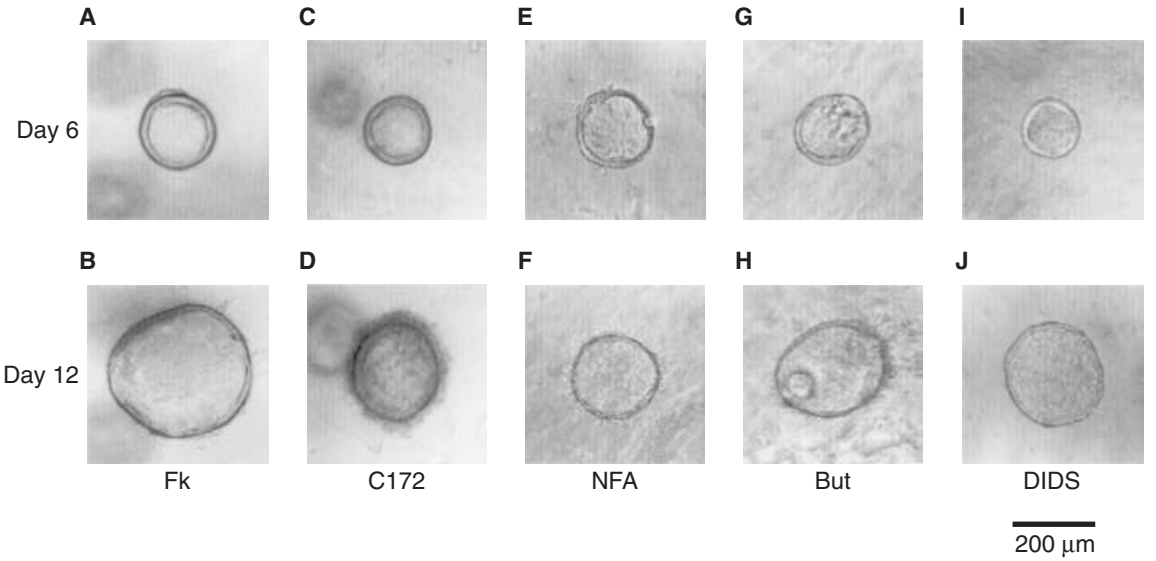

Fig. 1. Photomicrographs of cysts. The images show cysts before (*A*, *C*, *E*, *G*, and *I*) and after (*B*, *D*, *F*, *H*, and *J*) treatment with blockers of transepithelial ion transport in the presence of forskolin (10 μmol/L). Forskolin (Fk, 10 μmol/L) (A and B), $CFTR_{inh}$-172 (C172; 10 μmol/L) (C and D), niflumic acid (NFA; 100 μmol/L) (E and F), bumetanide (But; 100 μmol/L) (G and H), and DIDS (200 μmol/L) (I and J). Photomicrographs were taken on day six after seeding gels (A, C, E, G, and I) and day 12 after seeding gels (B, D, F, H, and J). Bar = 200 μm.

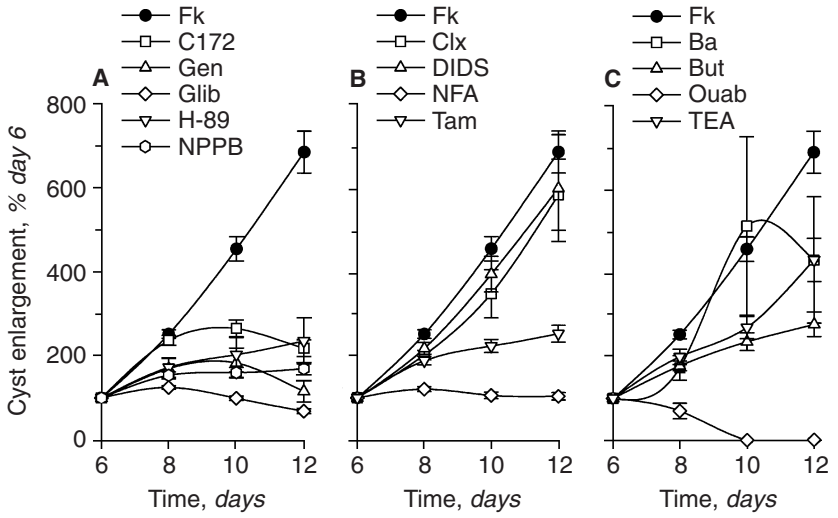

Fig. 2. Time course of cyst growth in the presence of blockers of transepithelial ion transport. Data show the relationship between cyst volume and time for CFTR blockers: $CFTR_{inh}$-172 (C172; 10 μmol/L; $N = 141$), genistein (Gen, 100 μmol/L; $N = 34$), glibenclamide (Glib, 100 μmol/L; $N = 26$), NPPB (50 μmol/L; $N = 22$), and H-89 (10 μmol/L; $N = 36$) (*A*); inhibitors of other types of apical membrane Cl^- channels: calix[4]arene (Clx, 10 μmol/L; $N = 31$), DIDS (200 μmol/L; $N = 32$), niflumic acid (NFA, 100 μmol/L; $N = 39$), and tamoxifen (Tam, 10 μmol/L; $N = 39$) (*B*); blockers of basolateral membrane ion channels and transporters: barium (Ba; 1 mmol/L; $N = 30$), bumetanide (But, 100 μmol/L; $N = 24$), ouabain (Ouab, 1 μmol/L; $N = 25$), and TEA (10 mmol/L; $N = 30$) (*C*). In A to C, filled circles show the effect of forskolin (Fk, 10 μmol/L; $N = 186$) on cyst growth. Data are expressed as a percentage of the value at day six, and are mean ± SEM of N observations, where N is the number of cysts. Different drugs were tested in the presence of forskolin (10 μmol/L) in at least three different experiments. Where not shown, error bars are smaller than symbol size.

(10 mmol/L). Bumetanide inhibits the Na^+-K^+-$2Cl^-$ cotransporter, ouabain inhibits the Na^+-K^+ ATPase, while barium and TEA inhibit many different types of K^+ channels.

Figure 1 shows images of cysts grown in the absence and presence of drugs from different classes of transport inhibitors, while Figure 2 quantifies the effects of drugs on cyst growth. Figure 1C and D and Figure 2A demonstrate that inhibitors of the CFTR Cl^- channel diminished the enlargement of cysts. After six days of treatment with $CFTR_{inh}$-172 (10 μmol/L), genistein (100 μmol/L), glibenclamide (100 μmol/L), H-89 (10 μmol/L) and NPPB (50 μmol/L), the volumes of cysts were significantly smaller than those treated with forskolin alone (day 12 data: $P < 0.01$; Student unpaired t test; Fig. 2A). Figure 1E, F, I, and J and Figure 2B show that blockers of other types of apical membrane Cl^- channels had variable effects on cyst growth. After six days treatment, niflumic acid (100 μmol/L) and tamoxifen (10 μmol/L) diminished (day 12 data: $P < 0.01$), while calix[4]arene (10 μmol/L) and DIDS (200 μmol/L) were without effect (day 12 data: $P > 0.05$). Figure 1G and H and Figure 2C

demonstrate that inhibitors of basolateral membrane ion channels and transporters diminished the enlargement of cysts with variable potency. After four days of treatment, ouabain (1 μmol/L) caused the disappearance of cysts (Fig. 2C). After six days of treatment, barium (1 mmol/L), bumetanide (100 μmol/L), and TEA (10 mmol/L) inhibited cyst growth (day 12 data: bumetanide, $P < 0.01$; barium and TEA, $P < 0.05$; Fig. 2C). These data suggest that blockers of CFTR and basolateral membrane ion channels and transporters inhibit cyst growth. They also suggest that drugs that block apical membrane Cl^- channels have variable effects on cyst growth, with those drugs that are without effect on the CFTR Cl^- channel failing to inhibit cyst growth.

Effects of the specific CFTR inhibitor $CFTR_{inh}$-172 on cyst formation

The observation that CFTR blockers diminish cyst growth suggests that these drugs might also inhibit cyst formation. To test this possibility, we grew cysts in the continuous presence of the specific CFTR inhibitor $CFTR_{inh}$-172 (10 μmol/L) from day 0 onward. Figure 3A and B show images of cysts grown in the absence and presence of $CFTR_{inh}$-172 (10 μmol/L) at day six, and Figure 3C and D quantify data for cyst volume and number. The data demonstrate that $CFTR_{inh}$-172 decreased not only the volume of individual cysts, but also the number of cysts formed. These data suggest that $CFTR_{inh}$-172 might inhibit both the formation and growth of renal cysts.

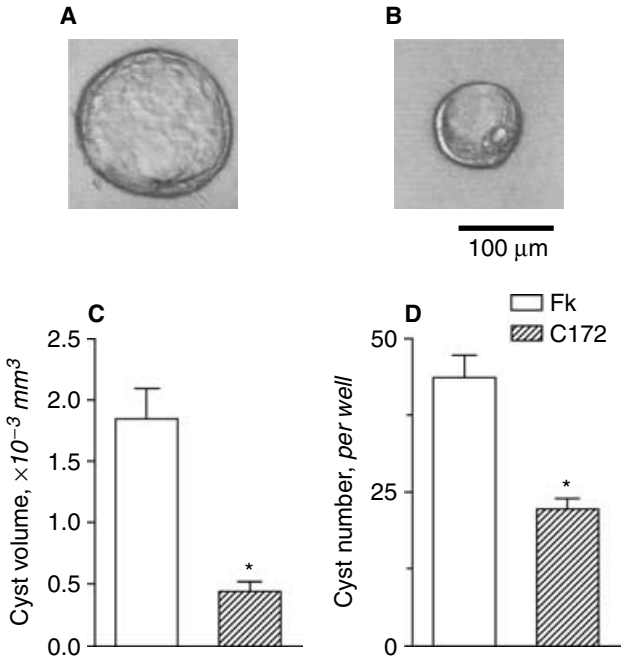

Fig. 3. The specific CFTR inhibitor $CFTR_{inh}$-172 inhibits the formation of MDCK cysts. (*A* and *B*) are images of MDCK cysts grown in collagen gels in the presence of forskolin (10 μmol/L) on day six after seeding the gel with MDCK cells. Control (A), cyst grown in the continuous presence of $CFTR_{inh}$-172 (10 μmol/L) (B). (*C* and *D*) show volume and number of cysts formed in the absence and presence of $CFTR_{inh}$-172 (10 μmol/L). Data are mean ± SEM (N = 23–186 cysts from 4–23 wells) made on day six. Asterisks indicate values that are significantly different from controls ($P < 0.05$).

Effects of inhibitors of transepithelial ion transport on cAMP-stimulated Cl^- current in MDCK epithelia

To evaluate the effects of drugs on fluid accumulation within cysts, we employed the Ussing chamber technique to record cAMP-stimulated Cl^- current, a measure of transepithelial Cl^- secretion. To enhance the magnitude of cAMP-stimulated Cl^- current, we grew MDCK epithelia at an air-liquid interface to augment the cell surface expression of CFTR [16]. In addition, we clamped transepithelial voltage at 0 mV, and imposed a large Cl^- concentration gradient across epithelia to magnify the size of cAMP-stimulated Cl^- current. For studies of drugs that inhibit Cl^- channels located in the apical membrane, the basolateral membrane was permeabilized with nystatin (0.36 mg/mL). However, for studies of drugs that inhibit basolateral membrane ion channels and transporters, the basolateral membrane was intact.

Figure 4A demonstrates that addition of forskolin (10 μmol/L) to the apical and basolateral solutions generated large Cl^- currents in nystatin-permeabilized epithelia ($\Delta I_{Cl}{}^{apical}$, 70 ± 8 μA cm^{-2}; $N = 69$; Fig. 4A, left and center), but small Cl^- currents in intact epithelia (ΔI_{sc}, 27 ± 2 μA cm^{-2}; $N = 16$; Fig. 4A, right). Following the activation of cAMP-stimulated Cl^- current, we added blockers of CFTR and other types of apical membrane Cl^- channels to the apical solution, and inhibitors of basolateral membrane ion channels and transporters to the basolateral solution. Figure 4 demonstrates that blockers of the CFTR Cl^- channel caused a significant reduction of cAMP-stimulated Cl^- current ($P < 0.01$; Student paired t test). With the exception of niflumic acid (100 μmol/L; $P < 0.01$), blockers of other types of apical membrane Cl^- channels failed to inhibit cAMP-stimulated Cl^- current ($P > 0.05$; Fig. 4). With the exception of TEA (10 mmol/L), inhibitors of basolateral membrane ion channels and transporters decreased cAMP-stimulated Cl^- current (Fig. 4). However, the reduction was only statistically significant for bumetanide (100 μmol/L; $P < 0.01$; $N = 5$). These data suggest that inhibition of CFTR-mediated fluid accumulation might account for some of the effects of drugs on cyst growth.

Inhibition of cell proliferation by blockers of transepithelial ion transport

In addition to fluid accumulation within the cyst lumen [3, 6], the growth of renal cysts involves cAMP-stimulated cell proliferation [4, 5]. To learn whether blockers of transepithelial ion transport also inhibit the

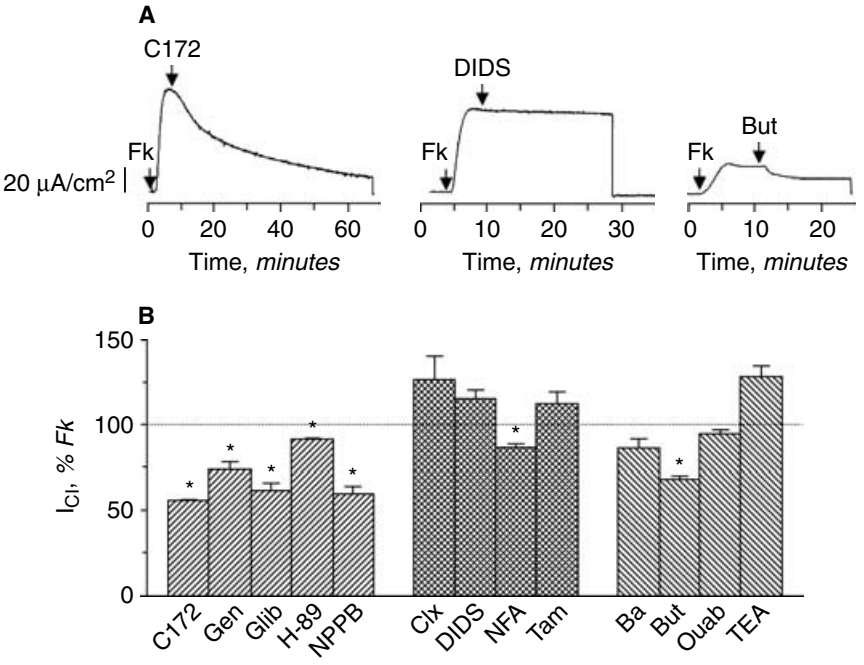

Fig. 4. Effect of blockers of transepithelial ion transport on cAMP-stimulated Cl^- current in MDCK epithelia. Representative recordings show the effects of $CFTR_{inh}$-172 (10 μmol/L), DIDS (200 μmol/L), and bumetanide (100 μmol/L) on cAMP-stimulated Cl^- current (*A*). To test the effects of $CFTR_{inh}$-172 and DIDS, we recorded I_{Cl}^{apical} after the basolateral membrane was permeabilized with nystatin (0.36 mg/mL), whereas to test the effect of bumetanide, we recorded I_{sc} with the basolateral membrane intact. Transepithelial voltage was clamped at 0 mV, and there was a large Cl^- concentration gradient across the epithelium (basolateral [Cl^-], 149 mmol/L; apical [Cl^-], 14.8 mmol/L). At the times indicated by the arrows labeled Fk, forskolin (10 μmol/L) was added to both the apical and basolateral solutions. At the times indicated by the other arrows, $CFTR_{inh}$-172 (10 μmol/L) and DIDS (200 μmol/L) were added to the apical solution, and bumetanide (100 μmol/L) was added to the basolateral solution. Inhibition of cAMP-stimulated Cl^- current by CFTR blockers, inhibitors of other types of apical membrane Cl^- channels, and blockers of basolateral membrane ion channels and transporters (*B*). Data show the magnitude of current inhibition by $CFTR_{inh}$-172 (10 μmol/L; $N = 5$), genistein (100 μmol/L; $N = 5$), glibenclamide (100 μmol/L; $N = 5$), H-89 (10 μmol/L; $N = 4$), NPPB (50 μmol/L; $N = 5$), calix[4]arene (10 μmol/L; $N = 5$), DIDS (200 μmol/L; $N = 5$), niflumic acid (100 μmol/L; $N = 8$), tamoxifen (10 μmol/L; $N = 5$), barium (1 mmol/L; $N = 4$), bumetanide (100 μmol/L; $N = 5$), ouabain (1 μmol/L; $N = 5$) and TEA (10 mmol/L; $N = 6$). Data are mean ± SEM of *N* observations measured once inhibition had reached steady state (e.g., bumetanide) or 3500 seconds after current activation (e.g., $CFTR_{inh}$-172). The dotted line indicates the control value before the addition of drugs. The asterisks indicate values of current inhibition that are significantly different from the control value ($P < 0.01$).

growth of MDCK cysts by retarding cell proliferation, we investigated the effects of drugs on the proliferation of nonpolarized MDCK cells in the presence of forskolin (10 μmol/L).

Figure 5A, C, and E shows the time course of cell proliferation in the absence and presence of forskolin (10 μmol/L) and blockers of transepithelial ion transport using MDCK media containing 0.01% FBS. In contrast to ADPKD cells [4, 5], forskolin was without effect on the proliferation of MDCK cells. In both the absence and presence of forskolin, the number of MDCK cells increased dramatically over a six-day period with very similar time courses (Fig. 5A).

After six days of treatment in the presence of forskolin, with the exception of H-89 (10 μmol/L), blockers of the CFTR Cl^- channel potently inhibited the proliferation of MDCK cells (day 6 data: $P < 0.01$; Student unpaired *t* test; Fig. 5A). Similarly, with the exception of calix[4]arene (10 μmol/L), blockers of other types of apical membrane Cl^- channels potently inhibited cell proliferation (day 6 data: $P < 0.01$; Student unpaired *t* test; Fig. 5C). However, inhibitors of basolateral membrane ion channels and transporters retarded cell proliferation with variable potency. Figure 5E demonstrates that ouabain (1 μmol/L) dramatically inhibited cell proliferation ($P < 0.01$), barium (1 mmol/L) and TEA (10 mmol/L) slowed (day 6 data: $P < 0.01$), but bumetanide (100 μmol/L) was without effect on cell proliferation (day 6 data: $P > 0.05$).

Our data indicate that many, but not all blockers of transepithelial ion transport inhibit the proliferation of nonpolarized MDCK cells in the presence of 0.01% FBS and forskolin (10 μmol/L). As controls, we performed several additional experiments. First, we used FBS (10%) because this was the FBS concentration in the media employed for cyst growth studies, and because FBS is known to alter both the rate of cell proliferation and the effects of drugs [11, 30]. Figure 5B, D, and F shows the time course of cell proliferation in the absence and presence

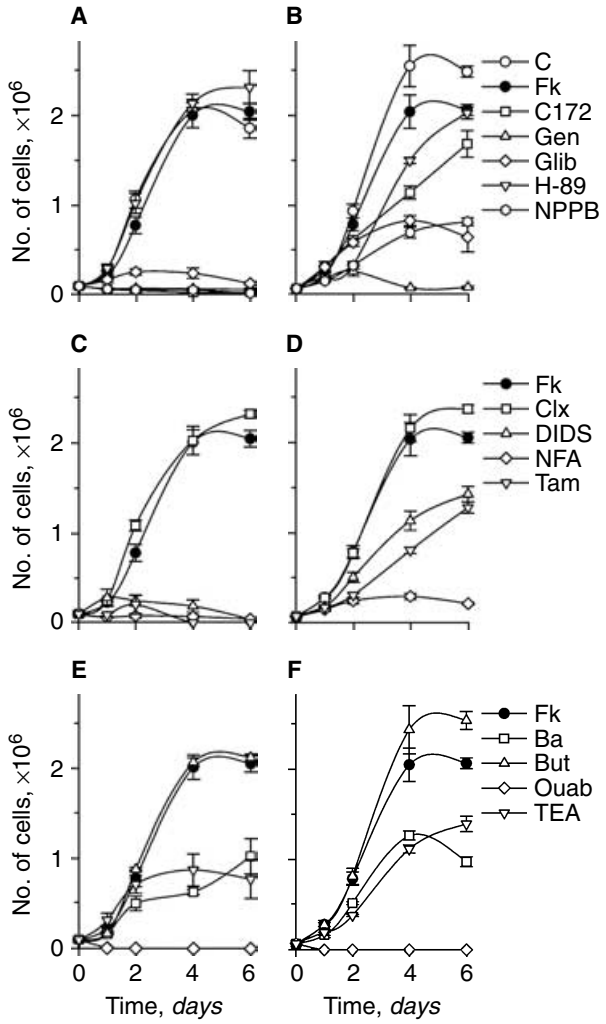

Fig. 5. Effects of blockers of transepithelial ion transport on the proliferation of nonpolarized MDCK cells. Data show the relationship between cell proliferation and time for blockers of transepithelial ion transport using MDCK media containing 0.01% FBS (*A*, *C*, and *E*) and 10% FBS (*B*, *D*, and *F*). CFTR blockers (A and B): $CFTR_{inh}$-172 (10 μmol/L; $N = 4$), genistein (100 μmol/L; $N = 4$), glibenclamide (100 μmol/L; $N = 4$), NPPB (50 μmol/L; $N = 4$), and H-89 (10 μmol/L; $N = 4$). Inhibitors of other types of apical membrane Cl^- channels (C and D): calix[4]arene (10 μmol/L; $N = 4$), DIDS (200 μmol/L; $N = 4$), niflumic acid (100 μmol/L; $N = 4$), and tamoxifen (10 μmol/L; $N = 4$). Blockers of basolateral membrane ion channels and transporters (E and F): barium (1 mmol/L; $N = 4$), bumetanide (100 μmol/L; $N = 4$), ouabain (1 μmol/L; $N = 4$), and TEA (10 mmol/L; $N = 4$). The open circles in (A) and (B) and the filled circles (A to F) show cell proliferation in the absence (control, C; $N = 4$-6) and presence of forskolin (Fk; 10 μmol/L; $N = 7$-8), respectively. Data are mean ± SEM of *N* observations, where *N* is the number of wells of cells. Other details as in Figure 2.

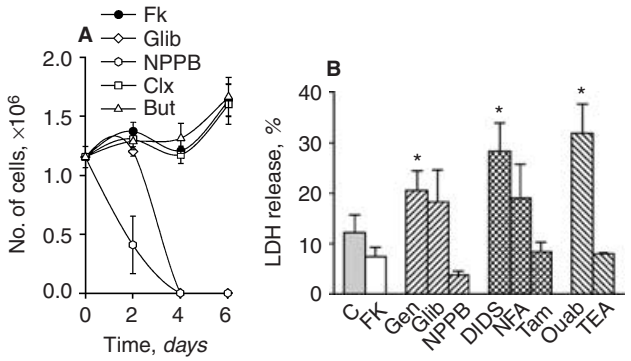

Fig. 6. Cell proliferation in polarized epithelia and cytotoxicity of drugs. Time course of cell proliferation in polarized MDCK epithelia in the absence and presence of glibenclamide (100 μmol/L), NPPB (50 μmol/L); calix[4]arene (10 μmol/L), and bumetanide (100 μmol/L) (*A*). Data are mean ± SEM ($N = 5$), where *N* is the number of epithelia. Different drugs were tested in at least two different experiments in the presence of forskolin (10 μmol/L). Other details as in Figure 2. Cytotoxicity of transepithelial ion transport inhibitors (*B*). Data show the release of LDH from nonpolarized MDCK cells grown in the absence and presence of CFTR blockers (genistein, 100 μmol/L; glibenclamide, 100 μmol/L; NPPB, 50 μmol/L), blockers of other types of apical membrane Cl^- channels (DIDS, 200 μmol/L; niflumic acid, 100 μmol/L; tamoxifen, 10 μmol/L), and inhibitors of basolateral membrane ion channels and transporters (ouabain, 1 μmol/L; TEA, 10 mmol/L). All inhibitors of transepithelial ion transport were tested in the presence of forskolin (10 μmol/L). Data are mean ± SEM (control, C, and forskolin, Fk, 10 μmol/L; $N = 9$; all other drugs $N = 6$). The asterisks indicate values that are significantly increased compared with the forskolin value ($P < 0.01$).

of forskolin (10 μmol/L) and blockers of transepithelial ion transport using MDCK media containing 10% FBS. Under these conditions, forskolin (10 μmol/L) retarded cell proliferation (day 6 data: $P < 0.01$; Student unpaired *t* test; Fig. 5B). When compared with the 0.01% FBS data, with the exception of H-89 (10 μmol/L), genistein (100 μmol/L), barium (1 mmol/L), TEA (10 mmol/L), and ouabain (1 μmol/L), all the blockers inhibited MDCK cell proliferation less potently in the presence of 10% FBS (Fig. 5B, D, and F). The weakening of inhibition of cell proliferation was especially pronounced for $CFTR_{inh}$-172 (10 μmol/L; Fig. 5B). Moreover, calix[4]arene (10 μmol/L) and bumetanide (100 μmol/L) stimulated the proliferation of MDCK cells (day 6 data: $P < 0.01$; Student unpaired *t* test; Fig. 5D and F).

Second, we tested the effects of blockers of transepithelial ion transport on MDCK cell proliferation in the absence of forskolin and presence of 0.01% FBS. With the exception of DIDS (100 μmol/L) and tamoxifen (10 μmol/L), which were without effect, all the drugs tested inhibited cell proliferation with similar potency to that observed in the presence of forskolin (data not shown).

Third, we tested the effects of some drugs on MDCK epithelia grown on permeable filter supports in the presence of 0.01% FBS. Once MDCK epithelia developed a high R_t (indicative of a polarized epithelium), we monitored the effects of drugs on cell proliferation in the presence of forskolin (10 μmol/L) over a six-day period. Figure 6A shows that the number of cells increased slowly in the presence of forskolin (10 μmol/L). They also show that glibenclamide (100 μmol/L) and NPPB (50 μmol/L) potently inhibited cell proliferation in polarized epithelia (day 6 data: $P < 0.01$; Student unpaired *t* test), while

bumetanide (100 μmol/L) and calix[4]arene (10 μmol/L) were without effect (day 6 data: $P > 0.05$; Fig. 6A). These data suggest that the effects of drugs on cell proliferation were not affected by cell polarity.

Finally, to test whether drugs that inhibited cell proliferation cause cell damage and death, we measured LDH release from nonpolarized MDCK cells cultured with 0.01% FBS for 24 hours in the absence and presence of forskolin and drugs. Figure 6B demonstrates that forskolin (10 μmol/L) was without effect on LDH release from MDCK cells ($P > 0.05$; Student unpaired *t* test). Moreover, glibenclamide (100 μmol/L), NPPB (50 μmol/L), niflumic acid (100 μmol/L), tamoxifen (10 μmol/L), and TEA (10 mmol/L) did not increase the release of LDH from MDCK cells compared with that observed in the presence of forskolin ($P > 0.05$; Fig. 6B). Only genistein (100 μmol/L), DIDS (200 μmol/L), and ouabain (1 μmol/L) augmented the release of LDH from MDCK cells ($P < 0.05$; Fig. 6B). These data suggest that blockers of the CFTR Cl^- channel might slow the proliferation of MDCK cells in the presence of forskolin without causing significant cytotoxic effects.

Relationship between cAMP-dependent cyst growth, cell proliferation, and Cl^- current

Our data suggest that inhibitors of the CFTR Cl^- channel diminish cyst growth by retarding both Cl^- secretion and cell proliferation. To evaluate this possibility, we examined cyst enlargement, Cl^- current, and cell proliferation in the presence of different inhibitors of transepithelial ion transport. Figure 7A demonstrates that cyst enlargement and cAMP-stimulated Cl^- current were well correlated in the presence of CFTR blockers (Pearson's correlation coefficient = 0.81; $r^2 = 0.66$; $P < 0.05$). Similar results were observed with all the drugs tested (Pearson's correlation coefficient = 0.69; $r^2 = 0.49$; $P < 0.01$; Fig. 7A). Figure 7B shows that cyst enlargement and cell proliferation (in the presence of 0.01% FBS) were not well correlated (CFTR blockers, Pearson's correlation coefficient = 0.44; $r^2 = 0.19$; $P > 0.05$; all drugs tested, Pearson's correlation coefficient = 0.43; $r^2 = 0.19$; $P > 0.05$). Finally, Figure 7C demonstrates that cyst enlargement and cell proliferation (in the presence of 10% FBS) were not well correlated for CFTR blockers (Pearson's correlation coefficient = 0.50; $r^2 = 0.25$; $P > 0.05$), but were well correlated for all the drugs tested (Pearson's correlation coefficient = 0.64; $r^2 = 0.39$; $P < 0.05$). These data suggest that CFTR blockers might retard cyst growth predominantly by inhibiting fluid accumulation within the cyst lumen.

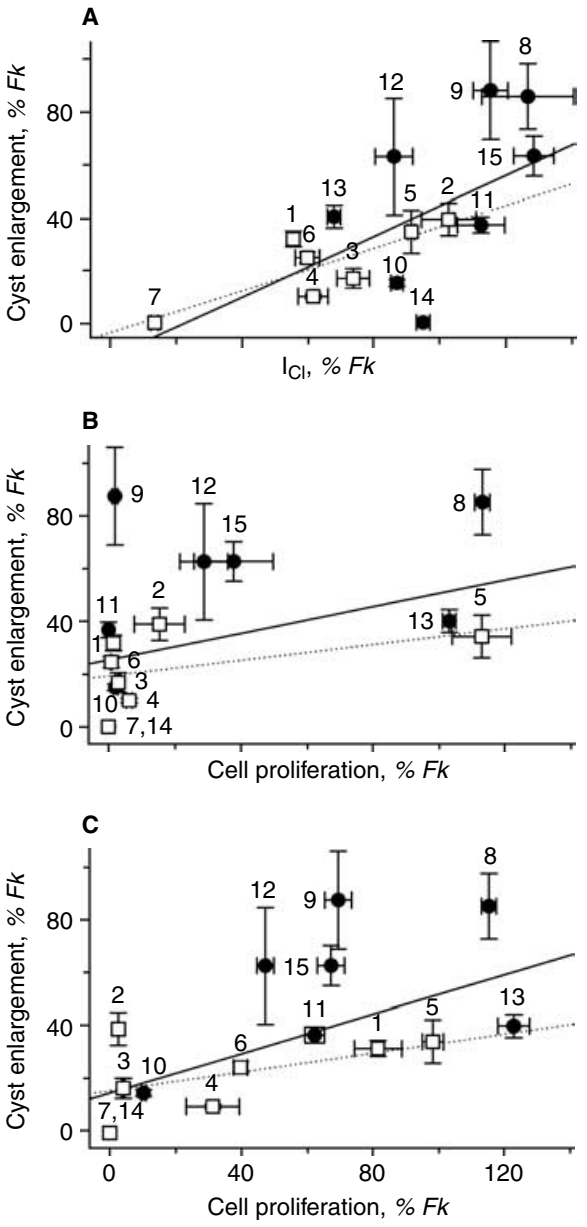

Fig. 7. Inhibition of cyst growth is correlated with inhibition of Cl^- current, but not inhibition of cell proliferation. Correlation between cyst enlargement and cAMP-stimulated Cl^- current (*A*). Correlation between cyst enlargement and cell proliferation in the presence of 0.01% FBS (*B*). Correlation between cyst enlargement and cell proliferation in the presence of 10% FBS (*C*). Key: 1, $CFTR_{inh}$-172 (10 μmol/L); 2, genistein (25 μmol/L); 3, genistein (100 μmol/L); 4, glibenclamide (100 μmol/L); 5, H-89 (10 μmol/L); 6, NPPB (50 μmol/L); 7, NPPB (400 μmol/L); 8, calix[4]arene (10 μmol/L); 9, DIDS (200 μmol/L); 10, niflumic acid (100 μmol/L); 11, tamoxifen (10 μmol/L); 12, barium (1 mmol/L); 13, bumetanide (100 μmol/L); 14, ouabain (1 μmol/L); and 15, TEA (10 mmol/L). Open squares represent CFTR blockers and filled circles denote other blockers of transepithelial ion transport. The continuous lines and the dotted lines show the fits of first-order regressions to all the data and to the data for the CFTR blockers, respectively. Data are mean ± SEM (cyst growth, $N = 20$-141; Cl^- current, $N = 5$-8; cell proliferation, $N = 4$).

DISCUSSION

The aim of our study was to test the effects of inhibitors of the CFTR Cl^- channel on renal cyst growth, and

identify their mechanism of action. For this purpose, we employed a battery of 12 inhibitors of transepithelial ion transport, including blockers of the CFTR Cl^- channel, inhibitors of other types of apical membrane Cl^- channels, and blockers of basolateral membrane ion channels and transporters. Using these agents, we investigated cyst growth, cAMP-stimulated Cl^- currents, and cell proliferation.

To test the effects of drugs on renal cyst growth, we employed type I MDCK cells. We chose MDCK cells because ADPKD tissue is scarce and difficult to culture, whereas MDCK cells are abundant and stable in culture [3]. However, type I MDCK cells are heterogeneous. For example, Orellana and Marfella-Scivittaro [31] identified four different clones of MDCK cells with variable cAMP responsiveness and distinct morphologic phenotypes. Like ADPKD cells [3, 4, 32], our MDCK cells form cysts in collagen gels and generate polarized epithelia with cAMP-stimulated Cl^- currents when grown on permeable filter supports. However, our MDCK cells differ from ADPKD cells in at least one important respect. Cyclic AMP agonists inhibit the proliferation of our MDCK cells, but stimulate that of ADPKD cells [4, 5]. Despite this difference, our MDCK cells are a valuable model system to examine the effects of inhibitors of transepithelial ion transport on renal cyst growth.

Using MDCK cells subcultured from a single cyst, Grantham et al [17] first demonstrated that agents that inhibit transepithelial ion transport retard cyst enlargement. The most potent inhibitors identified were ouabain and amiloride analogs that inhibit Na^+/Ca^{2+} exchange [17]. More recent studies have investigated the role of apical Cl^- channels and basolateral membrane K^+ channels in renal cyst growth. Hanaoka and Guggino [4] examined the effects of DIDS, diphenylamine-2-carboxylate (DPC), and glibenclamide on the growth of ADPKD cysts, while Sullivan et al [32] tested the hypothesis that glibenclamide retards the growth of ADPKD cysts by inhibiting ATP-sensitive K^+ channels located in the basolateral membrane of ADPKD epithelial cells. Importantly, these and other studies [8–10] raise the possibility that inhibitors of the CFTR Cl^- channel might diminish cyst growth.

In the present study, we demonstrate that all drugs that inhibit Cl^- transport by CFTR retard cyst growth. These drugs include agents that directly inhibit the CFTR Cl^- channel by either open-channel (e.g., glibenclamide [18]), or allosteric (e.g., genistein [21]) mechanisms. They also include agents that act indirectly either by inhibiting cAMP-dependent phosphorylation of CFTR (e.g., H-89 [24]), or by interfering with basolateral membrane ion channels and transporters that accumulate Cl^- within epithelial cells (e.g., bumetanide [33]). In contrast, DIDS and calix[4]arene, two drugs that inhibit other types of apical membrane Cl^- channels, but which are without effect on CFTR [26] [abstract; Singh AK et al, *Pediatr Pulmonol* 13(Suppl):237, 1996] failed to inhibit cyst growth. Similarly, Zn^{2+} (100 μmol/L), which blocks CLC-2, a member of the CLC family of voltage-gated Cl^- channels that is abundantly expressed in the kidney [27], was without effect on cyst growth (data not shown). The remaining blockers of other types of apical membrane Cl^- channels tested, tamoxifen and niflumic acid, inhibited cyst growth. However, these drugs are known to block the CFTR Cl^- channel [28, 29], raising the possibility that they inhibit cyst growth, at least in part, by blocking the CFTR Cl^- channel. Importantly, $CFTR_{inh}$-172, a potent, specific blocker of the CFTR Cl^- channel, retarded cyst formation and diminished cyst growth. We interpret our data to suggest that the CFTR Cl^- channel and basolateral membrane ion channels and transporters play key roles in renal cyst growth. Importantly, comparison of our data with previous work [4, 32] suggests that the pharmacology of cyst growth is similar in ADPKD and MDCK cells.

Previous work suggests that blockers of transepithelial ion transport diminish cyst enlargement by inhibiting fluid accumulation and/or cell proliferation [17, 32]. To investigate how CFTR inhibitors retard cyst enlargement, we examined cAMP-stimulated Cl^- secretion and cell proliferation.

To specifically examine the effects of drugs on apical membrane Cl^- channels, we adopted a strategy similar to that of Anderson and Welsh [25]. These authors investigated the different type of apical membrane Cl^- channels in airway and intestinal epithelia by permeabilizing the basolateral membrane with nystatin and imposing a large Cl^- concentration gradient across the epithelium [25]. An advantage of this approach is that the effects of inhibitors are more clearly observed when the magnitude of cAMP-stimulated Cl^- current is larger. Moreover, by employing a Cl^- concentration gradient, we mimicked the condition of gradient ADPKD cysts, which, like type I MDCK cells, are derived from the distal tubule [3]. Our data demonstrate that blockers of the CFTR Cl^- channel inhibit cAMP-stimulated Cl^- current. In contrast, with the exception of niflumic acid (for discussion, see above), blockers of other types of apical membrane Cl^- channels were without effect. Consistent with our data, Mangoo-Karim et al [33] demonstrated that DPC, an arylaminobenzoate that inhibits CFTR with low affinity [20], attenuates cAMP-stimulated I_{sc} in MDCK epithelia.

$CFTR_{inh}$-172 was the most potent inhibitor of cAMP-stimulated Cl^- currents among the drugs that we tested. However, the potency of $CFTR_{inh}$-172 observed in the present study is much less than that demonstrated by Ma et al [22]. A likely explanation for this difference is the rundown of cAMP-stimulated Cl^- currents in MDCK epithelia, which was pronounced over the prolonged time intervals required for $CFTR_{inh}$-172 to fully exert its

effects. When we corrected data for this rundown, the potency of CFTR blockers was reduced, while blockers of other types of apical membrane Cl^- channels appeared to potentiate cAMP-stimulated Cl^- current. A similar explanation might account for our failure to observe inhibition of cAMP-stimulated Cl^- currents with ouabain, barium, and TEA. Alternatively, the use of a large Cl^- concentration gradient might attenuate the inhibitory effects of these blockers. Consistent with this latter idea, when we bathed MDCK epithelia in symmetric NaCl-rich solutions, both ouabain and TEA inhibited cAMP-stimulated Cl^- currents, and the potency of bumetanide was increased [inhibition of I_{Cl}: bumetanide (100 μmol/L), 85 ± 6% ($N = 7$); ouabain (1 μmol/L), 58 ± 5% ($N = 4$), and TEA (10 mmol/L), 37 ± 13% ($N = 5$)].

Cyclic AMP agonists stimulate the proliferation of ADPKD epithelial cells [4, 5]. Therefore, we tested the effects of inhibitors of transepithelial ion transport on the proliferation of MDCK cells in the presence of forskolin. In contrast to the effects of forskolin on ADPKD epithelial cells, forskolin failed to stimulate the proliferation of our MDCK cells. One possible explanation for this result is that our MDCK cells endogenously generate high levels of intracellular cAMP to the extent that further stimulation by forskolin fails to augment cell proliferation. However, our observation that the PKA inhibitor H-89 was without effect on the proliferation of MDCK cells in the presence of 0.01% FBS argues against this possibility. Instead, our MDCK cells are reminiscent of normal human kidney cortex epithelial cells in which cAMP is either without effect or inhibits cell proliferation [4, 5]. An explanation for the effects of cAMP on the growth of MDCK cells is provided by the work of Yamada et al [34]. These authors suggest that cAMP inhibits the proliferation of MDCK cells by blocking the mitogenic effects of MAP kinases [34].

Blockers of K^+ channels inhibit cell proliferation by interfering with cell cycle progression [11]. In contrast, less is known about the effects of CFTR blockers on cell proliferation. In this study, we demonstrated that the CFTR blockers $CFTR_{inh}$-172, genistein, glibenclamide, and NPPB inhibit the proliferation of MDCK cells, and that the inhibitory effects were FBS-dependent. Two possible explanations for the effects of FBS are (*1*) FBS contains a variety of growth factors that stimulate cell proliferation [30], and (*2*) proteins in FBS might bind drugs to reduce their effective concentration [11]. Our observation that glibenclamide and NPPB caused the number of cells in MDCK epithelia to decrease to zero after four days of treatment suggests that these drugs either have cytotoxic effects or cause the detachment of MDCK cells from permeable filter supports. However, our studies using the LDH release assay argue that these drugs are without significant cytotoxic effects, a conclusion supported by previous work [35, 36]. Although our data do not reveal the targets with which glibenclamide and NPPB interact to inhibit cell proliferation, they suggest that these drugs retard cell cycle progression in MDCK cells. Consistent with this idea, these drugs have been demonstrated to arrest cancer cells at the G_0/G_1 stage of the cell cycle [12, 35].

Our data suggest that CFTR blockers diminish cyst growth by inhibiting both cAMP-stimulated Cl^- current and cell proliferation. To try to determine the relative importance of these effects of CFTR blockers for renal cyst growth, we performed a correlation test. We found that inhibition of cyst growth by CFTR blockers was correlated with inhibition of cAMP-stimulated Cl^- current, but not cell proliferation. Differences in the efficacy with which drugs inhibit cyst growth, cAMP-stimulated Cl^- current, and cell proliferation might be caused by a number of factors. These include (*1*) testing the effects of drugs on electrolyte transport using voltage-clamped epithelia, (*2*) using nonpolarized rather than polarized cells to examine the effects of drugs on cell proliferation, and (*3*) with the exception of $CFTR_{inh}$-172, the lack of specificity of the CFTR blockers tested. Despite these differences, our data suggest that fluid accumulation within the cyst lumen plays an important role in cyst enlargement. For example, DIDS, a drug that inhibited cell proliferation, but not cAMP-stimulated Cl^- current, failed to retard cyst growth. In contrast, bumetanide, a drug that inhibited cAMP-stimulated Cl^- current, but not cell proliferation, diminished cyst enlargement. Finally, the specific CFTR inhibitor $CFTR_{inh}$-172, which inhibited cAMP-stimulated Cl^- current more potently than cell proliferation, led to the formation of small cysts with thick walls (Fig. 1; for discussion see [17]). Thus, our data provide a cellular explanation for the effects of drugs on cyst growth.

The accessibility of basolateral membrane ion channels and transporters located on the outside (gel-facing) membrane of renal cysts explains why drugs that inhibit these ion channels and transporters diminish cyst growth. At first sight, the location of CFTR on the apical (lumen-facing) membrane of renal cysts argues that the CFTR Cl^- channel is an unsuitable target for drugs designed to retard cyst growth. However, knowledge of transepithelial Cl^- secretion and the molecular pharmacology of CFTR suggest otherwise (Fig. 8) [7, 20].

CFTR blockers are large organic anions [20]. When added to the outside of cysts, they enter epithelial cells by crossing the lipid phase of the basolateral membrane by nonionic diffusion. Once inside epithelial cells, the electrochemical gradient that drives transepithelial Cl^- secretion sweeps CFTR blockers toward the apical membrane, where they interact with CFTR. In contrast to other epithelial Cl^- channels [37], CFTR possesses a deep, wide intracellular vestibule, where open-channel blockers bind to occlude the channel pore and inhibit Cl^- permeation

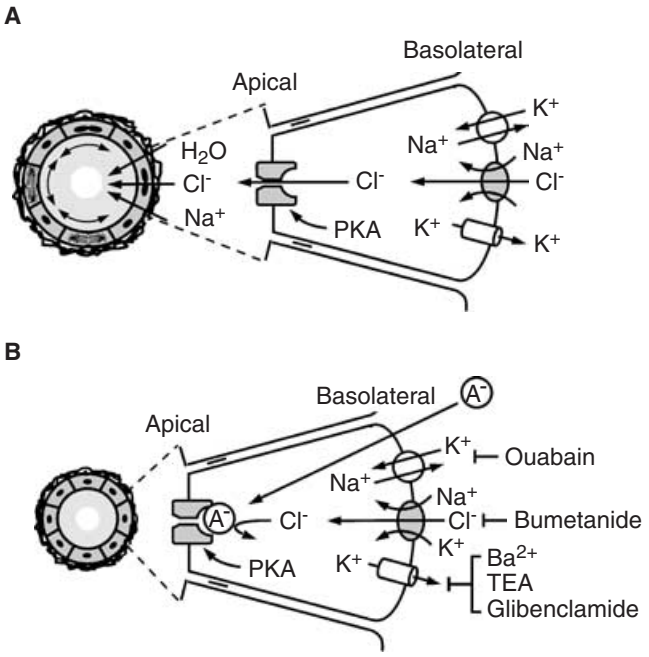

Fig. 8. Mechanism of inhibition of cyst growth by blockers of CFTR-driven Cl^- secretion. Model of Cl^- secretion by cyst-lining epithelial cells (*A*). Chloride movements drive fluid accumulation within the cyst lumen. Fluid accumulation causes cyst enlargement (*1*) directly by swelling cysts, and (*2*) indirectly by stretching cells (denoted by double-headed arrows) to promote cell division. Sites of inhibition of CFTR-driven Cl^- secretion (*B*). Different inhibitors of basolateral membrane ion channels and transporters and their targets are indicated. (In ADPKD epithelia, glibenclamide inhibits ATP-sensitive K^+ channels in the basolateral membrane and CFTR Cl^- channels in the apical membrane [32]). Open-channel blockers of CFTR (large organic anions; denoted by A^-) occlude the intracellular end of the CFTR pore to prevent Cl^- permeation. For clarity, only a model of the CFTR pore is shown. Genistein interacts with the nucleotide-binding domains to interfere with CFTR channel gating [21, 40]. The binding site of $CFTR_{inh}$-172 is currently unknown. See text and [7] for further details.

[18, 20]. Importantly, the interaction of open-channel blockers with CFTR is both voltage-dependent and sensitive to the external Cl^- concentration (for discussion, see [20]). Because of the basolateral to apical driving force for transepithelial Cl^- secretion, once open-channel blockers bind within the CFTR pore, they are likely to be difficult to dislodge. As a result, fluid accumulation within the cyst cavity is inhibited and cyst growth retarded (Fig. 8).

A potential link between CFTR-driven transepithelial Cl^- secretion and cell proliferation in MDCK cysts is provided by the work of Tanner et al [38]. These authors demonstrated that stretching cysts stimulates DNA synthesis in MDCK cells. This suggests that by attenuating fluid accumulation within the cyst lumen, CFTR blockers might inhibit the proliferation of MDCK cells (Fig. 8). Alternatively, CFTR blockers might inhibit the function of CFTR as a regulator of cell cycle progression [15]. By analogy with the human EAG K^+ channel [39], CFTR might alter its biophysical properties during the cell cycle to control cell volume. Alternatively, CFTR might regulate cell cycle progression by controlling the activity of the ion channels and transporters that regulate cell volume. Consistent with this latter idea, Vennekens et al [13] showed that CFTR attenuates the activity of VRACs by a mechanism independent of CFTR activation by cAMP-dependent phosphorylation. Thus, CFTR blockers might inhibit cell proliferation by blocking the actions of CFTR as a channel regulator.

CONCLUSION

The goal of this study was to investigate the effects of blockers of the CFTR Cl^- channel on renal cyst growth and identify their mechanism of action. Using type I MDCK cells as a model of cyst development and growth, we demonstrated that CFTR blockers and inhibitors of basolateral membrane ion channels and transporters retard cyst growth. By examining the effects of drugs on transepithelial Cl^- secretion and cell growth, we showed that CFTR blockers retard cyst growth principally by inhibiting transepithelial Cl^- secretion, not cell proliferation.

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