

An Extract from the Medicinal Plant *Phyllanthus acidus* and Its Isolated Compounds Induce Airway Chloride Secretion: A Potential Treatment for Cystic Fibrosis

Marisa Sousa, Jiraporn Ousingsawat, Roswitha Seitz, Supaporn Puntheeranurak, Ana Regalado, André Schmidt, Tiago Grego, Chaweewan Jansakul, Margarida D. Amaral, Rainer Schreiber, and Karl Kunzelmann

Institut für Physiologie, Universität Regensburg, Regensburg, Germany (J.O., R.S., S.P., R.S., K.K.); Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisboa and Centre of Human Genetics, National Institute of Health Dr. Ricardo Jorge, Lisboa, Portugal (M.S., A.R., A.S., T.G., M.D.A.); and Department of Physiology, Prince of Songkla University, Hat-Yai, Thailand (C.J.)

Received April 4, 2006; accepted October 25, 2006

ABSTRACT

According to previous reports, flavonoids and nutraceuticals correct defective electrolyte transport in cystic fibrosis (CF) airways. Traditional medicinal plants from China and Thailand contain phytoflavonoids and other bioactive compounds. We examined herbal extracts of the common Thai medicinal euphorbiaceous plant *Phyllanthus acidus* for their potential effects on epithelial transport. Functional assays by Ussing chamber, patch-clamping, double-electrode voltage-clamp and Ca^{2+} imaging demonstrate activation of Cl^- secretion and inhibition of Na^+ absorption by *P. acidus*. No cytotoxic effects of *P. acidus* could be detected. Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl^- secretion by increasing both intracellular Ca^{2+} and cAMP. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid. Additional experiments in human airway cells and CF transmem-

brane conductance regulator (CFTR)-expressing BHK cells and *Xenopus laevis* oocytes confirm the results obtained in native tissues. Cl^- secretion was also induced in tracheas of CF mice homozygous for Phe508del-CFTR and in Phe508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects defective electrolyte transport in CF airways by parallel mechanisms including 1) increasing the intracellular levels of second messengers cAMP and Ca^{2+} , thereby activating Ca^{2+} -dependent Cl^- channels and residual CFTR- Cl^- conductance; 2) stimulating basolateral K^+ channels; 3) redistributing cellular localization of CFTR; 4) directly activating CFTR; and 5) inhibiting ENaC through activation of CFTR. These combinatorial effects on epithelial transport may provide a novel complementary nutraceutical treatment for the CF lung disease.

Cystic fibrosis (CF) is an autosomal recessive disease with high frequency among the white population. It is caused by mutations in the cystic fibrosis transmembrane conductance

regulator (CFTR) gene. One mutation alone, Phe508del-CFTR, is present in at least one allele in approximately 90% of CF patients (Bobadilla et al., 2002). CF is characterized by deficient Cl^- transport and enhanced airway Na^+ absorption, mediated by epithelial Na^+ channels (ENaC) along with other abnormalities in ion transport. Pharmacological interventions attempt to correct defective ion transport among other pulmonary phenotypes. Recent strategies make use of natural food components because of their ready accessibility and low toxicity (deCarvalho et al., 2002; Bjarnsholt et al., 2005; Egan et al., 2004). These compounds act in different ways, such as correcting the trafficking defect of mutant

This work was supported by the Bayerische Forschungsförderung, Mukoviszidose e.V., the Else Kröner-Fresenius Stiftung, Deutsche Forschungsgemeinschaft SFB699 and by the research grant POCTI/MGI/47382/2002 and Centro de Investigação em Genética Molecular Humana (Fundação do Ministério de Ciência e Tecnologia de Portugal, Portugal and Fondo Europeo de Desarrollo Regional, European Union).

R.S. and K.K. share senior authorship. M.S. and J.O. contributed equally to the present work.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.025262.

ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na^+ channels; 8-SPT, 8-sulphophenyltheophylline; DPC-PX, 1,3-dipropyl-8-cyclopentylxanthine; DMEM, Dulbecco's modified Eagle's medium; DHBA, 2,3-dihydroxybenzoic acid (hypogallic acid); V_{te} , transepithelial voltage; R_{te} , transepithelial resistance; I_{sc} , short-circuit current; BHK, baby hamster kidney; wt, wild-type; IBMX, 3-isobutyl-1-methylxanthine; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; CPA, cyclopiazonic acid; MRS2179, 2'-deoxy- N^6 -methyl adenosine 3',5'-diphosphate; U73122, 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; NFA, niflumic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid; AM, acetoxymethyl ester; CCH, carbachol.

CFTR or potentiating residual CFTR activity (Kunzelmann and Mall, 2003; Moran and Zegarra-Moran, 2005; Van Goor et al., 2006).

Medicinal plants have been the basis for traditional pharmacology for many centuries. Around 500 different herb-based medicines have been counted in Thailand. They are used for the treatment of a variety of diseases such as cardiovascular failure, diabetes and cancer. In addition, their diuretic, anti-inflammatory, antiasthmatic, and antihypertensive properties are exploited, and some are used as dietary supplements and in sport medicine (Mueller-Oerlinghausen et al., 1971; Panthong et al., 1986). An increasing number of Thai medicinal plants have been taken to laboratories for purification and analysis. Through this approach, a number of novel compounds have been identified (Kanchanapoom et al., 2001; Wolfender et al., 2001). The extract of the traditional medicinal plant *Phyllanthus acidus* (mayom) has been shown to be enriched with adenosine (Fig. 1) (Cohen et al., 1997). Therefore, we have assessed the effects of this extract on the adenosine receptor system in mouse airways and in human airway epithelial cells. In particular, effects on A_1 and A_{2B} receptors were examined using pharmacological inhibitors 8-SPT, alloxazine, and DPC-PX. Stimulation of these receptors has been demonstrated to activate both Ca^{2+} -dependent and cAMP (CFTR)-regulated Cl^- channels and to affect the epithelial Na^+ channel ENaC, whereas others were unable to detect effects of adenosine on Cl^- secretion in CF tissues (Clancy et al., 1999). Apart from adenosine, *P. acidus* also contains other components that are likely to affect electrolyte transport in the airways, such as the flavonoid kaempferol and 2,3-dihydroxybenzoic acid (DHBA) (Illek and Fischer, 1998; Li and Wang, 2004). We compared the effects of *P. acidus* with the effect of commercially purchased adenosine, kaempferol, and DHBA and dissected out the underlying signaling pathways and the conductances affected. The present data indicate that extracts from *P. acidus* activate electrolyte secretion in epithelial tissues by means of intracellular second messengers and by directly increasing membrane expression and activity of ion channels. Thus, medicinal plant extracts from *P. acidus* may represent a novel and effective tool to correct defective electrolyte transport in CF.

Materials and Methods

Preparation of the Extract. Leaves of *P. acidus* were collected in Songkhla Province, Thailand. Fresh leaves were simmered at 60°C for 3 h in water. The clear solution of the extract was simmered at 50°C to reduce its volume to 50%, followed by partition extraction with water-saturated *n*-butanol. The *n*-butanol phase was collected and evaporated in vacuo and lyophilized. The extract was further purified by column chromatography as described previously (Jansakul et al., 1999). Identification was made by Prof. P. Sirirugsa (Department of Biology, Faculty of Science, Prince of Songkla University) and Prof. K. Hostettmann (Laboratoire de Pharmacognosie et Phytochimie, Ecole de Pharmacie, Université de Genève, Genève, Switzerland). The amounts of adenosine, kaempferol, and hypogallate acid (DHBA) present in the extract were 355.9 mg, 697.4 mg, and 1004.6 mg, respectively. Multiple lots of the extract were prepared and used for experiments. The extract was used at concentrations of 1 to 100 μ g/ml.

Ussing Chamber Recordings. Tracheas were removed from normal mice (C57BL/6; Charles River Laboratories, Sulzfeld, Ger-

many; animal facility University of Queensland) and mice homozygous for Phe508del-CFTR mice (Prof. Dr. B. Scholte, Institute of Cell Biology and Genetics, The Erasmus University Rotterdam, The Netherlands) after sacrificing the animals by cervical dislocation. After removing connective tissues, tracheas were opened by a longitudinal cut. Tissues were put immediately into an ice-cold buffer solution of the following composition: 145 mM NaCl, 3.8 mM KCl, 5 mM D-glucose, 1 mM $MgCl_2$, 5 mM HEPES, and 1.3 mM calcium gluconate. The tissues were mounted into a perfused micro Ussing

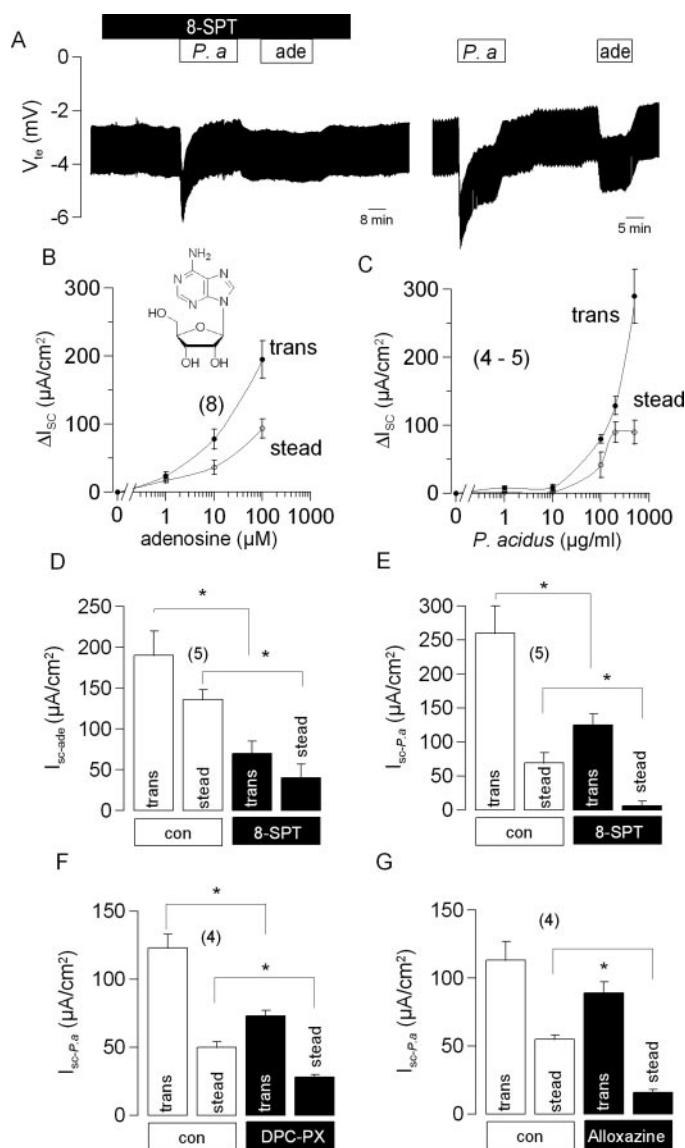


Fig. 1. Activation of ion transport by *P. acidus* extract via stimulation of adenosine receptors in mouse trachea. A, original Ussing chamber recording of the transepithelial voltage V_{te} in mouse trachea. Effects of *P. acidus* (*P. a*, 100 μ g/ml) and adenosine (*ade*, 100 μ M) in the presence or absence of the nonselective inhibitor of adenosine receptors, 8-SPT (10 μ M). B, concentration-dependent activation of steady-state (stead) and transient (trans) short-circuit currents (I_{sc}) by luminal application of adenosine. C, concentration-dependent activation of steady-state and transient short-circuit currents (I_{sc}) by luminal application of *P. acidus*. D, summary of transient and steady state I_{sc} induced by adenosine in the absence or presence of 8-SPT. E, summary of transient and steady state I_{sc} induced by *P. acidus* in the absence or presence of 8-SPT. F, summary of transient and steady state I_{sc} induced by *P. acidus* in the absence or presence of DPC-PX (200 nM). G, summary of transient and steady state I_{sc} induced by *P. acidus* in the absence or presence of alloxazine (10 μ M). *, significant difference (paired *t*-tests). Numbers in parentheses indicate number of experiments.

chamber with a circular aperture of 0.95 mm². Apical and basolateral surfaces of the epithelium were perfused continuously at a rate of 5 to 10 ml/min (chamber volume 2 ml). The bath solution contained 145 mM NaCl, 0.4 mM KH₂PO₄, 1.6 mM K₂HPO₄, 5 mM D-glucose, 1 mM MgCl₂, and 5 mM HEPES, and 1.3 mM calcium gluconate. pH was adjusted to 7.4, and all experiments were carried out at 37°C under open circuit conditions. Transepithelial resistance (R_{te}) was determined by applying short (1-s) current pulses ($I = 0.5 \mu A$) and the corresponding changes in transepithelial voltage (V_{te}) and basal V_{te} were recorded continuously. Values for V_{te} were referred to the serosal side of the epithelium. The equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law from V_{te} and R_{te} ($I_{sc} = V_{te}/R_{te}$).

Cell Culture. Human bronchial epithelial cells (16HBE14o-) and human CF airway epithelial cells homozygous for Phe508del-CFTR (CFBE) were kindly provided by Prof. Dr. D.C. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA) and were grown at 37°C in DMEM containing 4 mM D-glucose, 2 mM L-glutamine, 100 g/l fetal calf serum, 100 mg/l penicillin/streptomycin in an atmosphere of 5% CO₂ and 95% O₂. Transfected baby hamster kidney (BHK) cells were grown in the presence of 500 μM methotrexate.

Patch-Clamp. Cells were mounted on the stage of an inverted microscope (IM35; Zeiss, Oberkochen, Germany) and kept at 37°C. The bath was continuously perfused with Ringer's solution at a rate of 5 to 10 ml/min. Patch-clamp experiments were performed in fast whole-cell configuration. The patch pipettes had an input resistance of 2 - 4 M Ω when filled with a solution containing 30 mM KCl, 95 mM potassium gluconate, 1.2 mM NaH₂PO₄, 4.8 mM Na₂HPO₄, 1 mM EGTA, 0.726 mM CaCl₂, 1.034 mM MgCl₂, 5 mM D-glucose 5, and 1 mM ATP (32 mM Cl). The pH was adjusted to 7.2, and the Ca²⁺ activity was 0.1 μM . The access conductance was monitored continuously and was larger than 50 nS. Currents (voltage-clamp) and voltages (current-clamp) were recorded using a patch-clamp amplifier (EPC 7; List Medical Electronic, Darmstadt, Germany). Data were continuously stored on a computer hard disc. At regular intervals, membrane voltages were clamped in steps of 10 mV from -100 to +40 mV. Conductances were calculated according to Ohm's law.

Intracellular Ca²⁺ Concentration. For measurements of the intracellular Ca²⁺ concentration, cells were perfused with Ringer solution (145 mM NaCl, 0.4 mM KH₂PO₄, 1.6 mM K₂HPO₄, 5 mM glucose, 1 mM MgCl₂, 1.3 mM Ca²⁺-gluconate) at 37°C. Cells were loaded with 5 μM Fura-2 AM (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen) with 0.02% Pluronic (Invitrogen) for 1 h at room temperature. Fura-2 was excited at 340/380 nm, and emission was recorded between 470 and 550 nm using a charge-coupled device camera (CoolSnap HQ; Visitron Systems, Puchheim, Germany). Fluorescence was measured continuously using an inverted microscope IMT-2 (Olympus Deutschland GmbH, Hamburg, Germany) and a high speed polychromator system (VisiChrome; Visitron Systems). Experiments were controlled and analyzed using the software package Meta-Fluor (Molecular Devices, Sunnyvale, CA). All optical filters and dichroic mirrors were from AHF (Tübingen, Germany).

cRNAs for CFTR, ENaC Subunits, and P2Y₂. cDNAs encoding rat α, β, γ ENaC (kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland), wt-CFTR, Phe508del-CFTR, and the purinergic P2Y₂ receptor were linearized in pBlue-script with NotI or MluI and in vitro-transcribed using T7, T3, or SP6 promotor and polymerase (Promega, Madison, WI). After isolation from adult female *Xenopus laevis* frogs (Xenopus Express, Capetown, South Africa), oocytes were dispersed and defolliculated by a 45-min treatment with collagenase (type A; Roche, Mannheim, Germany). Subsequently, oocytes were rinsed and kept at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 2.5 mM sodium pyruvate, pH 7.55), supplemented with theophylline (0.5 mM) and gentamicin (5 mg/l).

Double Electrode Voltage-Clamp. Oocytes were injected with cRNA (1-10 ng) after dissolving in 47 nl of double-distilled water (Nanoliter Injector World Precision Instruments, Inc., Berlin, Germany). Water-injected oocytes served as controls. Two to 4 days after injection, oocytes were impaled with two electrodes (Harvard Bioscience, Edenbridge, UK) that had a resistance of <1 M Ω when filled with 2.7 M KCl. Using two bath electrodes and a virtual-ground head stage, the voltage drop across R_{series} was effectively zero. Membrane currents were measured by voltage-clamping of the oocytes (oocyte clamp amplifier; Warner Instruments, Hamden, CT) in intervals from -90 to +30 mV, in steps of 10 mV, each 1 s. Amiloride-sensitive conductances (G_{Amil}) were used in the present report to express the amount of whole-cell conductance that is inhibited by 10 μM amiloride. During the whole experiment, the bath was continuously perfused at a rate of 5 to 10 ml/min. All experiments were conducted at room temperature (22°C).

Viability Assay and Western blot. Twenty-four hours after seeding BHK cells, culture medium was changed, methotrexate was removed, and *Phyllanthus* extract was added. Forty-eight hours later, cells were collected, washed once with phosphate-buffered saline, re-suspended in bovine serum albumin solution (0.5 mg/ml in phosphate-buffered saline) and stained with calcein AM and ethidium homodimer-1 (LIVE/DEAD Viability/Cytotoxicity Kit; Invitrogen). Membrane-permeant calcein-AM (excitation/emission, 494/517 nm) is cleaved by esterases in living cells to yield cytoplasmic green fluorescence, whereas membrane-impermeant ethidium homodimer-1 (excitation/emission, 528/617 nm) labels nucleic acids of membrane-compromised cells with red fluorescence. Flow cytometry analysis was carried out at excitation of 488 nm in a FACScalibur flow cytometer, (BD Biosciences, San Jose, CA). For Western blot, cells were lysed after treatment, and 30 to 50 μg of total protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. Filters were probed with the anti-CFTR monoclonal antibody M3A7 (Chemicon, Temecula, CA).

Pulse-Chase and Immunoprecipitation Experiments. After treatment, cells were starved for 30 min in methionine-free DMEM (Invitrogen). Cells were then pulse-labeled for 30 min in the same medium with 150 $\mu Ci/ml$ [³⁵S]methionine (MP Biomedicals, Irvine, CA) as described previously (Farinha and Amaral, 2005). After chasing for 0, 0.5, 1, 2, and 3 h in DMEM supplemented with fetal bovine serum (Invitrogen) and 1 mM nonradioactive methionine, cells were lysed in 1 ml of radioimmunoprecipitation assay buffer and immunoprecipitated. In brief, samples were centrifuged at 14,000g for 30 min, and the supernatant was incubated overnight at 4°C with 1.5 μg of anti-CFTR M3A7 antibodies. Then, 25 μg of Protein-G agarose beads (Roche, Basel, Switzerland) were added for a further 4 h at 4°C; beads were washed four times using 1 ml of radioimmunoprecipitation assay buffer, and protein was eluted for 1 h at room temperature after addition of 80 μl of cracking buffer: 0.5 mM dithiothreitol (Sigma), 0.001% (w/v) bromophenol blue (Merck, Darmstadt, Germany), 5% (v/v) glycerol (Merck), 1.5% (w/v) SDS, and 31.25 mM Tris, pH 6.8. Samples were electrophoretically separated on 7% (w/v) polyacrylamide gels. Quantification of the core-glycosylated form of wt- or Phe508del-CFTR (band B) at a given chase time t was estimated as the percentage given by the ratio of the amount of the band B at that chase time (P) over its amount at chase time 0 (P_0) (i.e., at the end of the pulse period). Likewise, maturation efficiency was determined by the appearance of the fully glycosylated form (band C) also as a percentage given by the ratio of P , the amount of band C at time t , over P_0 , the amount of band B at the start of the chase ($t = 0$).

Iodide Efflux Assay. Iodide efflux experiments were performed by a standard protocol using an ion-selective electrode. In brief, cells were incubated for 1 h in loading buffer containing 136 mM NaI, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 11 mM glucose, and 20 mM HEPES, adjusted to pH 7.4 with NaOH. Cells were thoroughly washed with efflux buffer (136 mM NaNO₃ replacing NaI in the loading buffer) to remove extracellular iodide and then equilibrated in 2.5 ml of efflux

buffer for 1 min. The efflux buffer was changed at 1-min intervals. Four minutes after anion substitution, cells were exposed to 10 μM forskolin and 50 μM genistein for 4 min. The amount of iodide in each 2.5-ml sample of efflux buffer was determined using an iodide-selective electrode (Mettler Toledo, Columbus, OH). Cell loading and measurements were performed at room temperature.

Materials and Statistical Analysis. All compounds used were of the highest available purity. 3-Isobutyl-1-methylxanthine (IBMX), forskolin, ATP, adenosine, UDP, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid, suramin, DIDS, BAPTA-AM, amiloride, carbachol, cyclopiazonic acid (CPA), kaempferol, 8-SPT, dihydrobenzoic acid, alloxazine, DPC-PX, and MRS2179 were all from Sigma (Deisenhofen, Germany). U73122 was from Calbiochem (Nottingham, UK). Student's *t* test *P* values <0.05 were accepted to indicate statistical significance (*).

Results

Activation of Ion Transport by *P. acidus* Extract via Stimulation of Adenosine Receptors in Mouse Trachea. A major component of *P. acidus* is adenosine, which is known to activate ion transport in airway epithelial cells. We therefore compared the effects of adenosine and the *P. acidus* extract on electrolyte transport in mouse airways. Both adenosine (100 μM) and *P. acidus* (100 $\mu\text{g}/\text{ml}$) induced a transient and steady-state Cl^- secretion when applied to the luminal side of the epithelium (Fig. 1A). The effects of both adenosine and *P. acidus* were dose-dependent and did not saturate in the concentration range examined (Fig. 1, B and C). The nonselective inhibitor of adenosine receptors 8-SPT (10 μM) completely inhibited the effects of adenosine on ion transport. The secretory response of *P. acidus* was largely reduced and only part of the transient response remained (Fig. 1, A, D, and E). The transient and steady-state responses are probably due to activation of both Ca^{2+} - and cAMP-mediated Cl^- secretion. Mouse trachea is dominated by Ca^{2+} -activated Cl^- secretion but also contains cAMP-activated CFTR Cl^- channels. Stimulation of A_1 adenosine receptors increases cytosolic Ca^{2+} , whereas activation of $\text{A}_{2\text{B}}$ receptors enhances intracellular cAMP. The A_1 receptor antagonist DPC-PX (200 nM) inhibited both transient and steady-state I_{sc} activated by *P. acidus* extract, whereas the $\text{A}_{2\text{B}}$ receptor antagonist alloxazine (10 μM) blocked only steady-state responses (e.g., cAMP-mediated Cl^- secretion) (Fig. 1, F and G). Moreover, Cl^- secretion was also induced by *P. acidus* in mouse colonic ($\Delta I_{\text{sc}} = 61 \pm 7.3 \mu\text{A}/\text{cm}^2$; $n = 6$) and nasal ($\Delta I_{\text{sc}} = 151 \pm 17.3 \mu\text{A}/\text{cm}^2$; $n = 6$) native epithelia.

We also examined possible effects of the *P. acidus* extract on other purinergic receptors. Stimulation of luminal P_2Y_2 receptors with ATP or UTP (both 100 μM), or inhibition of P_2Y receptors with pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid or suramin (both 100 μM) did not interfere with the ability of *P. acidus* to induce Cl^- secretion. Moreover, a role of P_2Y_1 receptors in the effects of *P. acidus* on epithelial transport was unlikely, because *P. acidus* had similar effects in the presence of the specific P_2Y_1 agonist MRS2179 (10 μM) (data not shown). Taken together, our data indicate that activation of A_1 and $\text{A}_{2\text{B}}$ receptors contributes substantially to the observed induction of Cl^- secretion by *P. acidus* extract.

Activation of Ca^{2+} and cAMP-dependent Cl^- Secretion by *P. acidus* in Mouse Trachea. Luminal stimulation of mouse and human airways with adenosine increases in-

tracellular cAMP and leads to a steady CFTR-dependent Cl^- secretion (Huang et al., 2001). We found that the transient I_{sc} and a substantial part of the steady state I_{sc} activated by adenosine (100 μM) in mouse airways is inhibited by niflumic acid (NFA, 10 μM), an inhibitor of Ca^{2+} activated Cl^- channels (Fig. 2, A and B). Thus, adenosine activates both cAMP- and Ca^{2+} -dependent Cl^- secretion. Similar to adenosine, both transient and steady secretion induced by *P. acidus* were inhibited by NFA and DIDS, another blocker of Ca^{2+} -activated Cl^- channels (Fig. 2, C and D). We further examined whether *P. acidus* also activates cAMP-dependent CFTR Cl^- channels. To that end, we prestimulated mouse airways with IBMX (100 μM) and forskolin (2 μM) and found that Cl^- secretion induced by *P. acidus* was significantly

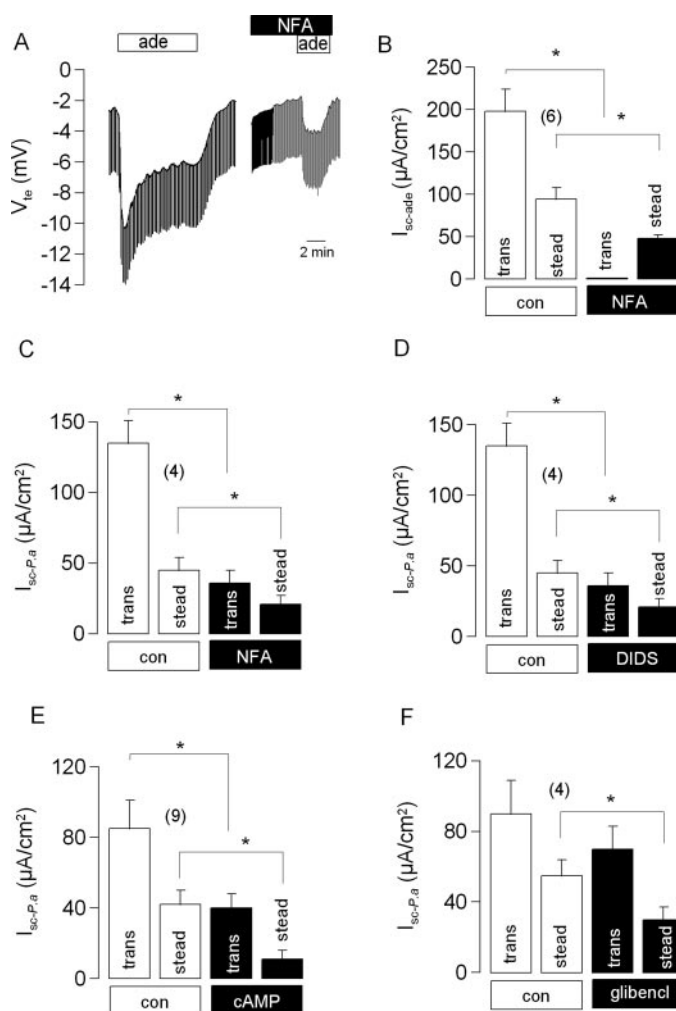


Fig. 2. Adenosine and *P. acidus* extract activate Cl^- secretion via increase of intracellular Ca^{2+} and cAMP in mouse trachea. A, original recordings of the effects of adenosine (ade, 100 $\mu\text{g}/\text{ml}$) in the absence or presence of the Cl^- channel blocker niflumic acid (NFA, 100 μM) in mouse trachea. B, summary of transient and steady state I_{sc} induced by adenosine in the absence or presence of NFA (100 μM). C, summary of the transient and steady-state I_{sc} activated by *P. acidus* in the absence or presence of NFA. D, summary of the transient and steady-state I_{sc} activated by *P. acidus* in the absence or presence of DIDS (100 μM). E, summary of the transient and steady-state I_{sc} activated by *P. acidus* in the absence or presence of prestimulation with IBMX (100 μM) and forskolin (2 μM) (cAMP). F, summary of the transient and steady-state I_{sc} activated by *P. acidus* in the absence or presence of glibenclamide (glibencl, 100 μM). *, significant difference (paired *t* test). Numbers in parentheses indicate number of experiments.

reduced. Moreover, application of the CFTR inhibitor glibenclamide also reduced steady-state I_{sc} induced by *P. acidus* (Fig. 2F). Thus, *P. acidus* activates two luminal Cl^- channels, CFTR and a Ca^{2+} -activated Cl^- channel of unknown molecular identity. The effects of *P. acidus* were not limited to mouse trachea; it also activated I_{sc} of $161 \pm 17.5 \mu A/cm^2$ ($n = 6$) and $48 \pm 8.7 \mu A/cm^2$ ($n = 6$) when applied to mouse nasal epithelium and proximal colon, respectively.

We further demonstrated that transient Cl^- secretion induced by *P. acidus* is due to an increase in intracellular Ca^{2+} . Thus, we emptied endoplasmic reticulum Ca^{2+} stores with $10 \mu M$ CPA, which induced a transient I_{sc} . In the presence of CPA, the transient Cl^- secretion induced by $100 \mu g/ml$ *P. acidus* was largely reduced (Fig. 3, A and B). Activation of Cl^- secretion was also reduced when intracellular Ca^{2+} was chelated with $10 \mu M$ BAPTA-AM or when phospholipase C was inhibited using U73122 ($10 \mu M$) (Fig. 3, C and D). Ca^{2+} signaling in airways is compartmentalized; i.e., basolateral stimulation of Cl^- secretion by carbachol (CCH; $100 \mu M$) activated Cl^- secretion through activation of basolateral K^+ channels, rather than luminal Ca^{2+} -activated Cl^- channels

(Huang et al., 2001). Thus, luminal application of *P. acidus* extract did not interfere with stimulation of basolateral M3 receptors by carbachol (Fig. 3, E and F). Moreover, basolateral application of *P. acidus* induced smaller effects on ion transport, compared with luminal application (Fig. 3G).

Activation of Cl^- Secretion by Kaempferol and DHBA. *P. acidus* extract contains the flavonoid kaempferol and DHBA (Li and Wang, 2004) (Fig. 4). Both compounds induced a dose-dependent Cl^- secretion, albeit smaller than that activated by adenosine (Fig. 4). Substantial amounts of the Cl^- secretion induced by kaempferol and DHBA were inhibited by the Cl^- channel blocker NFA. We then sought to determine whether the effects of *P. acidus* extract on epithelial ion transport could be reproduced by a mixture of the isolated components adenosine, kaempferol, and DHBA. As shown in Fig. 5, the mixture demonstrated similar, albeit larger effects than those produced by the *P. acidus* extract. Thus, a defined mixture of isolated components reproduces the effects of *P. acidus*.

***P. acidus* Activates Cl^- Secretion in Human Airway Epithelial Cells and Overexpressing Oocytes.** We ob-

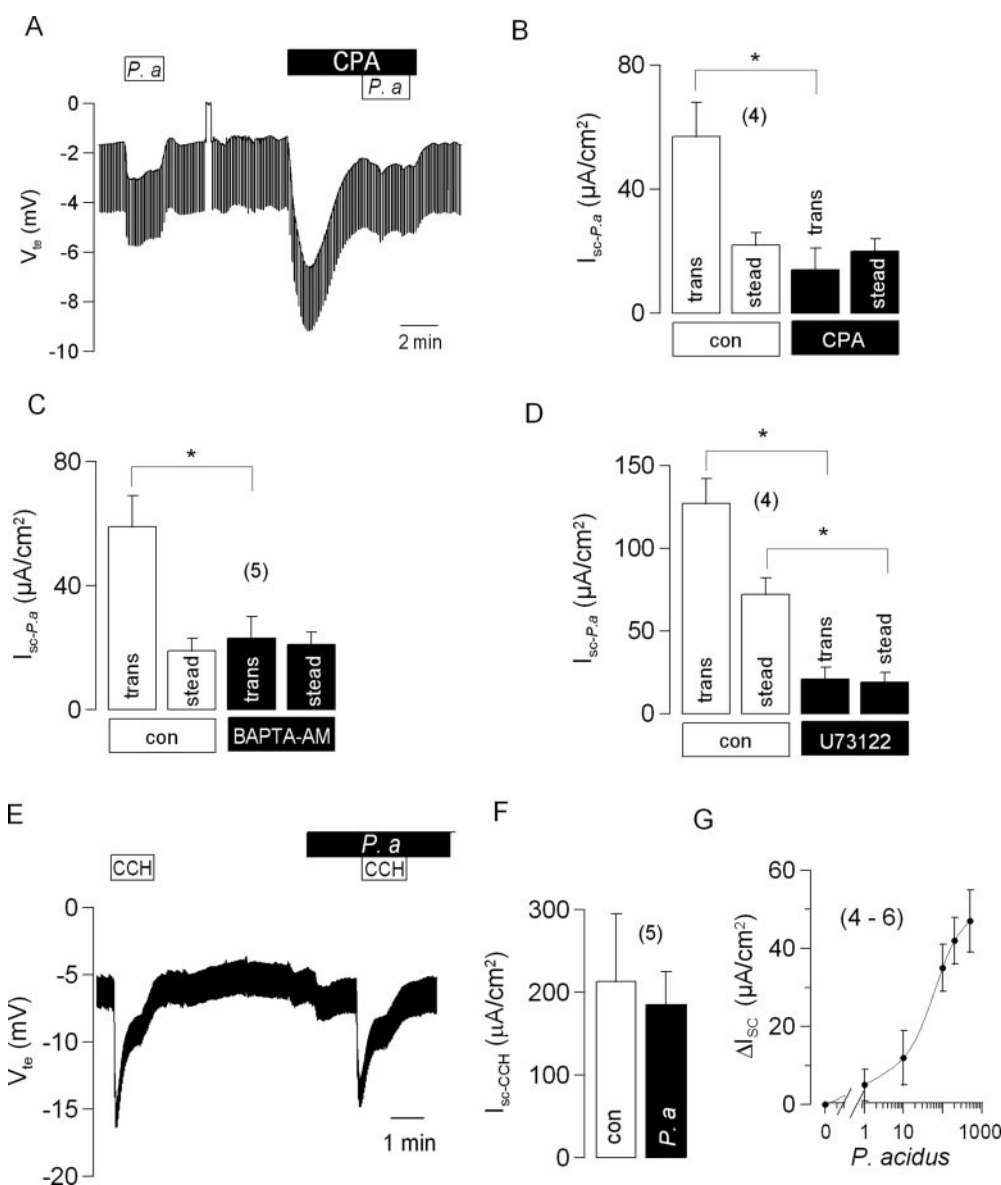


Fig. 3. *P. acidus* extract increases intracellular Ca^{2+} by luminal store depletion in mouse trachea. A, original Ussing chamber recordings of the effects of *P. acidus* (*P. a*, $100 \mu g/ml$) in mouse trachea, in the absence or presence of the Ca^{2+} ATPase inhibitor cyclopiazonic acid (CPA, $10 \mu M$). B, summary of the transient and steady state I_{sc} activated by *P. acidus* in the absence or presence of CPA. C, summary of the transient and steady state I_{sc} activated by *P. acidus* in the absence or presence of BAPTA-AM. D, summary of the transient and steady state I_{sc} activated by *P. acidus* in the absence or presence of the phospholipase C inhibitor U73122. E, original recording of the effects of basolateral stimulation with CCH ($100 \mu g/ml$) in mouse trachea, in the absence or presence of *P. acidus*. F, summary of the effects of CCH on I_{sc} in the absence or presence of the *P. acidus*. G, concentration-dependent activation of I_{sc} by basolateral stimulation with adenosine. * indicates significant difference (paired t test). #, significant difference compared with the effects of *P. acidus* (unpaired t test). Numbers in parentheses indicate number of experiments.

tained further evidence for the activation of Cl^- currents by *P. acidus* through whole-cell patch-clamp experiments with human airway epithelial cells (16HBE). The cells were voltage-clamped and exposed to $10 \mu\text{g/ml}$ *P. acidus*, which activated a whole-cell current. The effects of *P. acidus* extract were compared with those of the well known secretagogue ATP ($100 \mu\text{M}$) (Fig. 6, A and B). Whole-cell conductances activated by either *P. acidus* or ATP were inhibited by removal of Cl^- from the extracellular bath solution (data not shown). Intracellular Ca^{2+} concentrations were directly measured in 16HBE cells using the Ca^{2+} -sensitive dye Fura-2. As shown in Fig. 6, C and D, both ATP and *P. acidus* enhanced intracellular Ca^{2+} concentrations.

P. acidus also induced Cl^- secretion in overexpressing cells. Oocytes from *X. laevis* endogenously express Ca^{2+} -activated Cl^- channels. As shown in Fig. 7A, *P. acidus* ($100 \mu\text{g/ml}$) induced a transient Cl^- secretion, probably due to the activation of endogenous Ca^{2+} -activated Cl^- channels in *X.*

laevis oocytes. DIDS ($100 \mu\text{M}$) completely suppressed current activation by *P. acidus* (Fig. 7, A and B). In contrast to noninjected oocytes, where *P. acidus* only transiently activated Cl^- secretion, oocytes overexpressing wild-type (wt) CFTR exhibited both transient and steady-state Cl^- currents when exposed to *P. acidus* (Fig. 7C). Current activation was significant compared with the effects of the phosphodiesterase inhibitor IBMX (1 mM), which increases intracellular cAMP (Fig. 7, C and D). Thus, in *X. laevis* oocytes, *P. acidus* activates endogenous Ca^{2+} -activated Cl^- channels and overexpressed CFTR Cl^- channels. Numerous reports have demonstrated inhibition of ENaC during activation of CFTR. In fact, lack of ENaC inhibition by mutant CFTR has been proposed as a mechanism for enhanced Na^+ absorption in CF (Stutts et al., 1995). We thus coexpressed CFTR and the epithelial Na^+ channel ENaC in *X. laevis* oocytes and found amiloride-sensitive Na^+ currents under control conditions (Fig. 7E). Activation of Ca^{2+} -dependent and CFTR Cl^- currents by *P. acidus* ($100 \mu\text{g/ml}$) inhibited amiloride-sensitive Na^+ channels (Fig. 7, E and F). Moreover, inhibition of ENaC was not observed by *P. acidus* when CFTR was inhibited by the specific inhibitor 172, thus showing that ENaC currents were not directly inhibited by *P. acidus*. In other words, *P. acidus* had no direct effect on Na^+ currents in the absence of CFTR activity.

We further examined whether *P. acidus* also activates mutant CFTR, which carries the most common mutation Phe508del. Stimulation of Phe508del-CFTR with IBMX (1 mM) and forskolin ($2 \mu\text{M}$) activated a small but significant

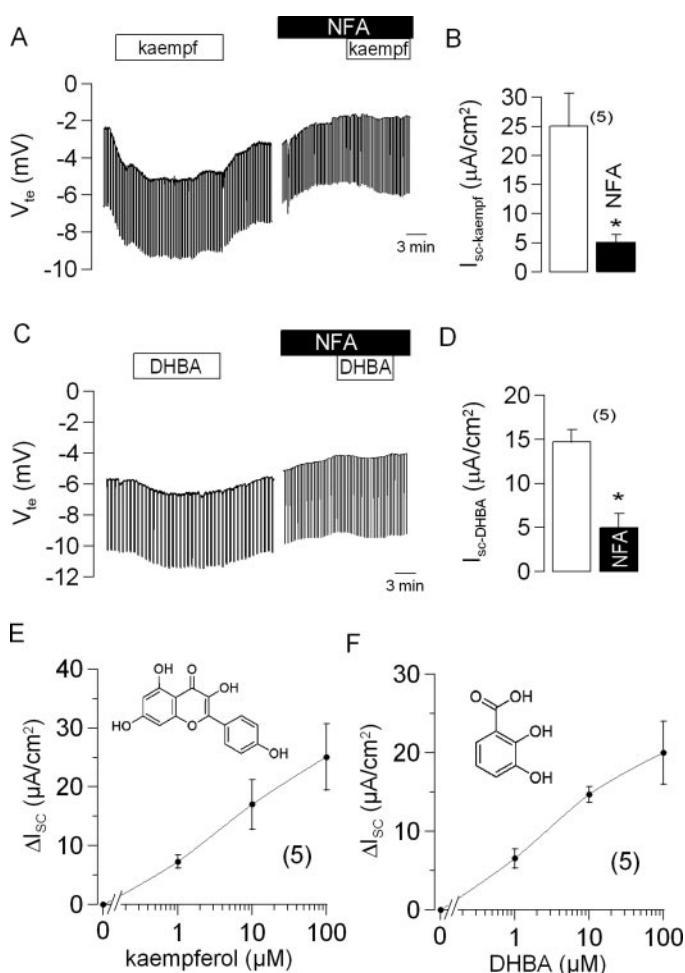


Fig. 4. The *P. acidus* components kaempferol and DHBA activate Cl^- secretion in mouse trachea, which is partially inhibited by NFA. A, original Ussing chamber recording of the effects of kaempferol ($100 \mu\text{M}$) in the absence or presence of NFA ($100 \mu\text{M}$). B, summary of the I_{sc} activated by kaempferol in the absence or presence of NFA ($100 \mu\text{M}$). C, original recording of the effects of DHBA ($100 \mu\text{M}$) in the absence or presence of NFA ($100 \mu\text{M}$). D, summary of the I_{sc} activated by DHBA in the absence or presence of NFA. E, concentration-dependent activation of steady state I_{sc} by luminal application of kaempferol. F, concentration-dependent activation of steady state I_{sc} by luminal application of DHBA. * indicates significant difference (paired t test). Numbers in parentheses indicate number of experiments.

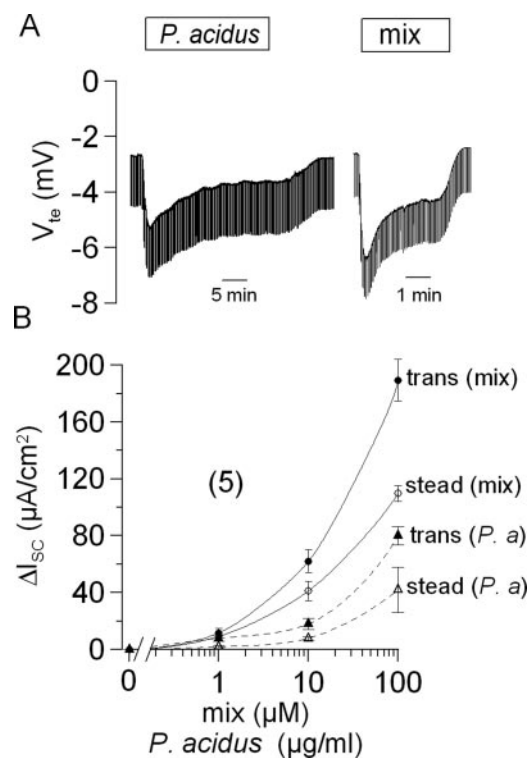


Fig. 5. *P. acidus* extract and coapplication of isolated components have similar effects on ion transport in mouse trachea. A, original recordings show the effects of *P. acidus* extract and of a mixture of the pure *P. acidus* components: adenosine, kaempferol and DHBA. B, summary of the transient (trans, filled symbols) and steady state (stead, open symbols) I_{sc} activated by *P. acidus* (dashed line) or the mixture of components (solid line). *, significant difference (paired t test). Numbers in parentheses indicate number of experiments.

Cl⁻ current in *X. laevis* oocytes. Activation of Phe508del-CFTR currents was significantly augmented after incubation of the oocytes with *P. acidus* (100 µg/ml) for 24 h. This was not observed when oocytes were incubated in control Ringer solution (Fig. 7, G–I). Moreover, in preliminary experiments with Phe508del-CFTR overexpressing BHK cells, Phe508del-CFTR was membrane-rescued by low temperature (26°C). In these cells, *P. acidus* induced a large I⁻ efflux, suggesting activation of mutant CFTR by *P. acidus* (data not show). This result suggests that membrane-rescued Phe508del-CFTR can be activated by *P. acidus*. Moreover, *P. acidus* may increase membrane expression by redistribution of Phe508del-CFTR from intracellular compartments (Lim et al., 2004).

***P. acidus* Is Not Toxic for Mammalian Cells and Acts As a Potentiator of CFTR.** Using a viability/cytotoxicity test (see *Materials and Methods*) we examined whether *P. acidus* exerts any toxic effect on mammalian cells (Fig. 8). After 48-h incubation with *P. acidus* in the 50–200 µg/ml concentration range, BHK cells stably expressing wt-CFTR or Phe508del-CFTR were analyzed by flow cytometry. Graphs represent bivariate frequency distributions of red-fluorescent (585 nm) ethidium homodimer-1-stained dead cell population (y-axis, arbitrary units) over green-fluorescent (530 nm) calcein-stained live cell population (x-axis, arbitrary units). The fraction of live cells was larger than 95% under all conditions, indicating that *P. acidus* is not toxic for mammalian cells, up to a concentration of 200 µg/ml (Fig. 8, A and B). The cells continued to divide normally in the presence of the extract (data not shown).

We examined steady-state levels of expression of wt-CFTR and Phe508del-CFTR in BHK cells by Western blot and found no significant changes after incubation with *P. acidus*

extract up to a concentration of 200 µg/ml. Only wt-CFTR levels were slightly increased after 48 h incubation with *P. acidus*. In metabolic pulse-chase experiments, we examined the turnover rate of the core-glycosylated form (band B) of wt-CFTR and Phe508del-CFTR, which was not affected by

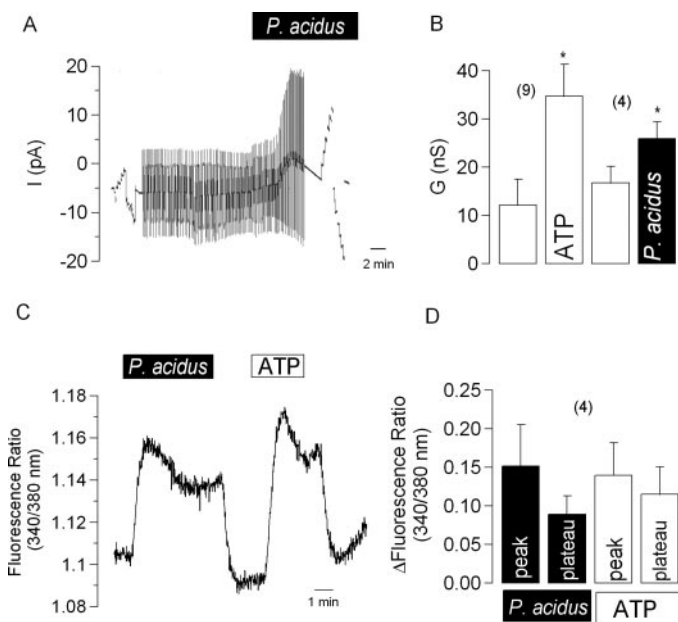


Fig. 6. *P. acidus* extract activates Cl⁻ secretion and increases intracellular Ca²⁺ in human airway epithelial cells. A, activation of a whole-cell current in a human airway epithelial (16HBE) by *P. acidus* (100 µM). B, summary of the effects of *P. acidus* and comparison with the effects of ATP (100 µM) on whole-cell conductance (G_m). C, original recording of the 340/380 fluorescence ratio (cytosolic Ca²⁺ concentration) and effects of *P. acidus* and ATP. D, summary of the 340/380 fluorescence ratio changes induced by *P. acidus* and ATP. *, significant difference (paired *t* test). Numbers in parentheses indicate number of experiments.

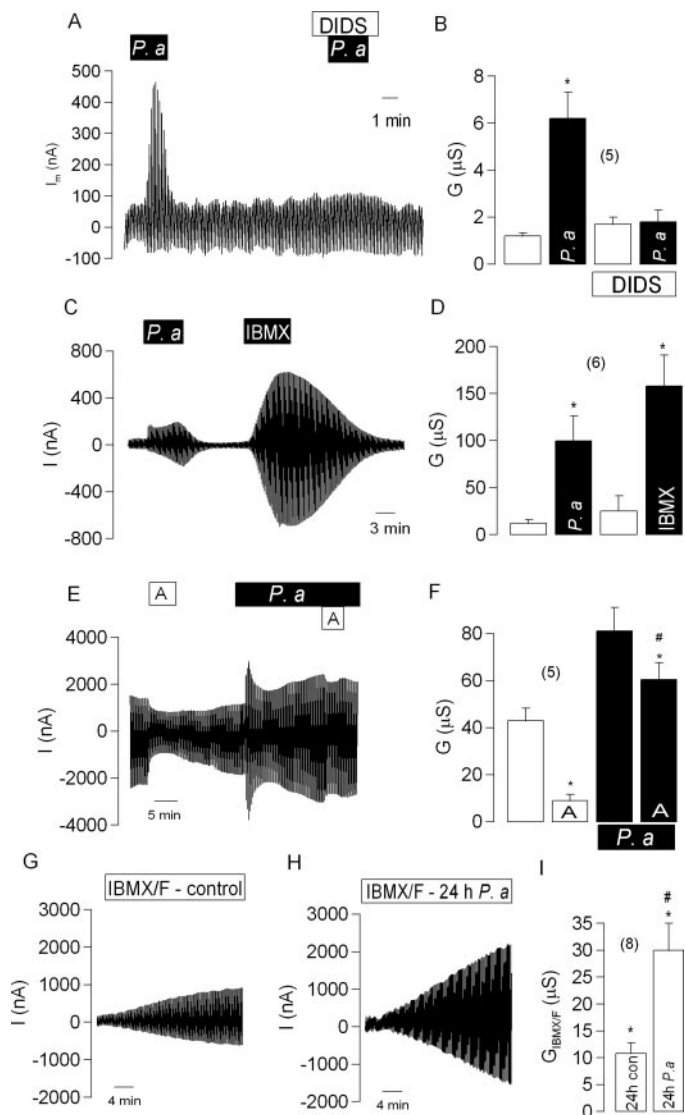


Fig. 7. *P. acidus* extract activates CFTR- and Ca²⁺-dependent Cl⁻ conductance and inhibits Na⁺ conductance in *X. laevis* oocytes. A, current recording from a noninjected *X. laevis* oocyte obtained by double-electrode voltage clamp. Transient activation of endogenous Ca²⁺-activated Cl⁻ currents by *P. acidus* (100 µg/ml) and effects of DIDS (100 µM). B, summary of the effects of *P. acidus* in the absence or presence of DIDS. C, current recording from a CFTR-expressing *X. laevis* oocyte. Activation of nontransient CFTR whole-cell currents by *P. acidus* and IBMX (1 mM). D, summary of the effects of *P. acidus* and IBMX in CFTR expressing oocytes. E, current recording from a CFTR/ENaC coexpressing *Xenopus* oocyte. Inhibition of Na⁺ conductance (ENaC) by amiloride (A, 10 µM) and reduced effects of amiloride after stimulation of CFTR by *P. acidus*. F, summary of the effects of amiloride before and after activation of CFTR by *P. acidus*. G, activation of whole-cell currents by IBMX and forskolin (F) in Phe508del-CFTR expressing *X. laevis* oocytes after 24-h control incubation. H, activation of whole-cell currents by IBMX (1 mM) and forskolin (2 µM) in Phe508del-CFTR expressing *X. laevis* oocytes after 24-h incubation in *P. acidus* extract (100 µg/ml). I, summary of the whole-cell Cl⁻ conductance activated by IBMX/F in *P. acidus*-incubated oocytes or control oocytes. *, significant difference (paired *t* test). #, indicates significant difference of the effects of amiloride or IBMX/forskolin (F), respectively (paired *t* test). Numbers in parentheses indicate number of experiments.

100 $\mu\text{g/ml}$ *P. acidus* (Fig. 8, B and D). Moreover, *P. acidus* did not alter the efficiency of CFTR maturation (i.e., conversion of band B to band C when processing efficiency was assessed by densitometry) (Fig. 8C). To assess a potentiator effect of *P. acidus* on wt-CFTR when expressed in mammalian cells, we performed iodide efflux assays in BHK cells stably expressing Phe508del-CFTR, which was membrane-rescued by low temperature (26°C) (Denning et al., 1992). In fact, the forskolin/genistein-activated iodide efflux was enhanced after acute application of *P. acidus* (Fig. 8D). It is noteworthy that the delay of activation that is typically observed for Phe508del-CFTR was corrected by *P. acidus* (Fig. 8D), suggesting a correction of the gating defect of this most common CFTR-mutant.

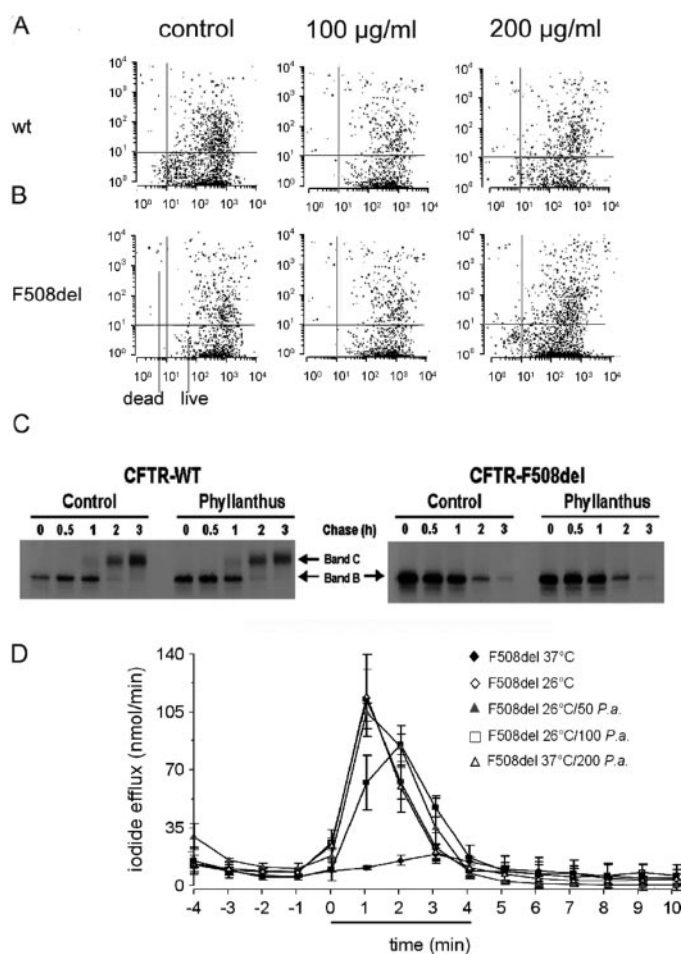


Fig. 8. *P. acidus* extract is not cytotoxic and activates Phe508del-CFTR. Flow cytometry viability assay of BHK cells stably expressing (A) wt-CFTR or (B) Phe508del-CFTR, after 48-h incubation with different concentrations of *P. acidus* as indicated in each panel. Graphs represent bivariate frequency distributions of red-fluorescent (585 nm) ethidium homodimer-1-stained dead cell population (y-axis, arbitrary units) over green-fluorescent (530 nm) calcein-stained live cell population (x-axis, arbitrary units). The population of live cells was larger than 95% in each assay. C, effect of *P. acidus* (50 $\mu\text{g/ml}$, 48 h) on the turnover and processing of wt- and Phe508del-CFTR. BHK cells stably expressing wt- or Phe508del-CFTR were pulse-labeled for 30 min, chased for the indicated times and lysates were immunoprecipitated with an anti-CFTR antibody. Panels show turnover of core-glycosylated CFTR (band B) of wt- and Phe508del-CFTR and appearance of fully glycosylated wtCFTR (band C). Each experiment was performed at least three times. D, 10 μM /50 μM , black line. Cells were grown at 37°C or 26°C, in the absence or presence of different concentrations of (*P.a.*) as indicated. Data indicate means \pm S.E.M. ($n = 4$).

We further examined whether *P. acidus* and its isolated components were able to activate Cl^- secretion in tracheas of CF mice homozygous for the most common CFTR-mutation Phe508del. Ussing chamber recordings demonstrated that adenosine; a mix of adenosine, kaempferol, and DHBA; and *P. acidus* activate Cl^- secretion in CF tracheas (Fig. 9, A and B). These results were further

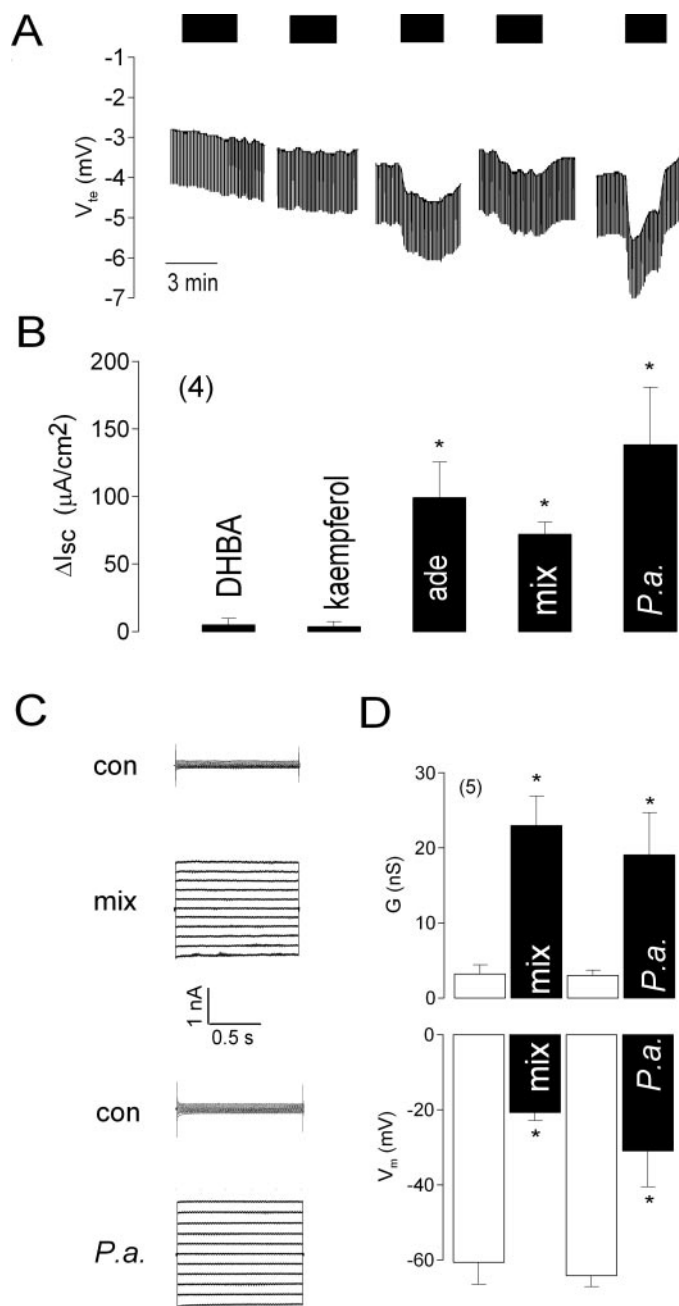


Fig. 9. *P. acidus* activates Cl^- secretion in CF tracheas and human CF airway epithelial cells. A, original Ussing chamber recordings of V_{te} in tracheas of mice homozygous for Phe508del-CFTR. Black boxes indicate effects of DHBA, kaempferol, adenosine, a mix of all three components and of extract. B, summary of the short circuit currents activated by the individual components, the mix of components and, C, whole cell current traces activated by a mix of the three isolated components and of extract. Cells were held at their membrane voltage and voltage-clamped ± 50 mV. D, summary of the whole-cell conductances activated by the mix and extract. *, significant difference (paired *t* test). Numbers in parentheses indicate number of experiments.

confirmed in patch-clamp experiments with human airway epithelial cells (CFBE) homozygous for Phe508del-CFTR. Both a mix of the individual components (each at 100 μ M) and *P. acidus* (100 μ g/ml) activated a whole cell Cl^- current and depolarized membrane voltages (Fig. 9, C and D). Taking these data together, we see that *P. acidus* exhibits multiple pro-secretory effects on epithelial electrolyte transport in normal and CF airways. The results strongly suggest that *P. acidus* acts as a potentiator of wtCFTR and Phe508del-CFTR. It may therefore represent a novel therapeutic strategy to circumvent the defect in electrolyte transport observed in CF epithelial tissues (Fig. 10).

Discussion

Complementary Treatment of CF Lung Disease by Nutraceuticals. Defective electrolyte transport is a major cause of severe lung disease in cystic fibrosis. Various therapeutic interventions have been developed to counteract abnormal ion transport caused by a lack of Cl^- secretion and hyperabsorption of electrolyte. Pharmacological strategies have been reviewed in several recent articles (Kunzelmann and Mall, 2003; Boucher, 2004; Kerem, 2005). A major step forward in identifying new therapeutic small molecules is high-throughput quantitative screening for CFTR activators (potentiators) and correctors (Ma et al., 2002; Pedemonte et al., 2005; Van Goor et al., 2006). Although bioactive molecules are discovered by this procedure, it is nevertheless both time and cost-intensive and may typically require 7 years or longer for analysis of the mechanism of action, evaluation, and preclinical and clinical testing before FDA approval is obtained. On the other hand, compounds that have already received FDA approval, such as phenyl butyrate or aminoglycosides, or common food components and plant constituents, could be tested for their potential therapeutic benefits, because they can be available much faster in the clinical setting.

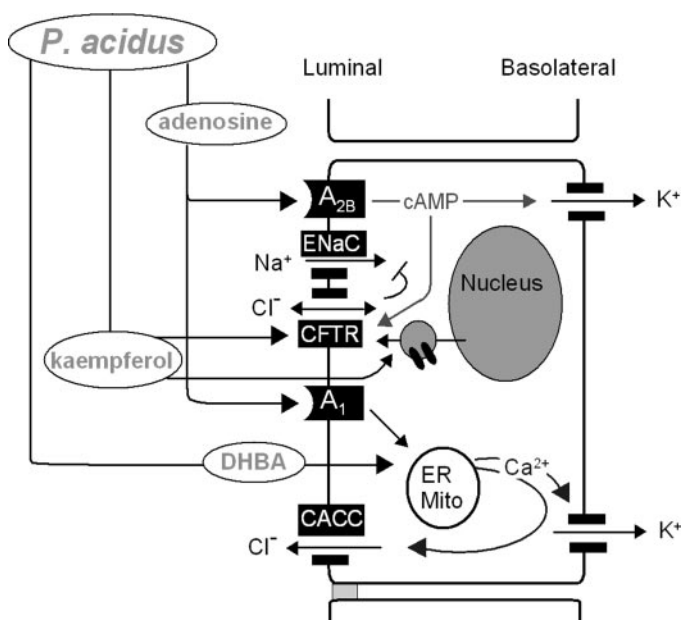


Fig. 10. Transport model of an airway epithelial cell and effects of *P. acidus* and its major constituents adenosine, kaempferol, and dihydrobenzoic acid (DHBH).

Phytoflavonoids such as genistein have been extensively tested and have been proven to activate CFTR (Hwang et al., 1997; Illek and Fischer, 1998; Mall et al., 2000; Suaud et al., 2001, 2002). Flavonoids also restore functional interactions between mutant Phe508del-CFTR or G551-CFTR and ENaC (Suaud et al., 2001). Genistein is currently under investigation in a phase I pilot study in coadministration with phenylbutyrate. Also other dietary flavonols, such as quercetin and kaempferol, have been identified as activators of Cl^- secretion (Cermak et al., 1998). The effects of the spice curcumin have been inconsistent among different groups who tested this compound, but are nevertheless currently under examination in a phase I clinical trial (Berger et al., 2004; Egan et al., 2004; Song et al., 2004). Another study has demonstrated opening of CFTR Cl^- channels by vitamin C (L-ascorbate) (Fischer et al., 2004). Vitamin C was identified as a biological regulator of CFTR-mediated Cl^- secretion. Although citrus limonoids were found to increase Cl^- conductance in epithelial cells to an extent comparable with that of genistein (deCarvalho et al., 2002), we were unable to detect significant effects of L-ascorbate in mouse trachea (data not shown). This is probably due to the relatively low levels of CFTR expression in this tissue.

Constituents of the Herbal Plant *P. acidus* Enhance Electrolyte Secretion. Plant extracts from *P. acidus* contain various bioactive compounds, such as adenosine, kaempferol, and hypogallic acid. The effects of these compounds include: 1) increasing the intracellular second messengers cAMP and Ca^{2+} and thereby activating CFTR- and Ca^{2+} -dependent Cl^- channels; 2) activating CFTR directly, as demonstrated for flavonoids; 3) increasing membrane expression of CFTR; 4) enhancing the driving force for luminal Cl^- exit by activating basolateral K^+ channels; and 5) reducing ENaC activity through activation of CFTR, thereby reducing NaCl absorption and preventing dehydration of the airway surface liquid (Fig. 10).

The components of *P. acidus* have been shown to affect membrane ion transport in previous studies. Apart from activating CFTR directly, flavonoids have also been shown to inhibit endoplasmic reticulum Ca^{2+} -ATPase and to stimulate mitochondrial Ca^{2+} uptake (Montero et al., 2004), which may affect endoplasmic reticulum chaperones and thus CFTR membrane traffic. Flavonoids also lead to a favorable redistribution of Phe508del-CFTR within cellular compartments, without directly affecting processing of the protein (Lim et al., 2004). This may explain why *P. acidus* had only modest effects on biogenesis of CFTR, but activated Phe508del-CFTR currents after incubation of oocytes or short-term application to Phe508del-CFTR-expressing BHK cells. Adenosine and other xanthines have been found to bind and activate mutant and wt-CFTR directly. Moreover, adenosine activates purinergic A_1 and A_{2B} receptors, thereby increasing intracellular Ca^{2+} and cAMP. Hypogallic acid induced Ca^{2+} -dependent Cl^- secretion, an effect that had been demonstrated for caffeic acid, another component of *P. acidus* (Lin et al., 2004).

Previous studies demonstrated that the P2Y receptor agonist ATP had only short-term effects on ion transport in the airways, due to inactivation by rapid hydrolysis. It is unlikely that the effects of *P. acidus* are short lasting, because its components are more stable and will probably not be removed from the airway surface as effectively.

Subsequent studies in a mouse model will have to compare the effects of local versus systemic application. These studies should also examine pharmacokinetics of absorption and pharmacodynamics of these compounds, which are currently not known.

Ethnopharmacology—A New Source for CF Therapeutics? The present study identified bioactive components in herbal extracts of *P. acidus*. In a recent elegant study, a growth-deficient yeast strain was used as a drug discovery surrogate bioassay to identify natural plant products restoring Cl^- channel function (deCarvalho et al., 2002). During the course of this study, limonoids were identified as Phe508del-CFTR correctors. In previous studies with the extract from another medicinal plant, *Randia siamensis*, we also found effects on ion transport properties in mouse trachea (Jansakul et al., 1999). Extracts from *R. siamensis* induced Cl^- secretion by activation of Ca^{2+} -dependent Cl^- channels. Similar to *P. acidus*, *R. siamensis* also contains flavonoids and other bioactive compounds, such as pseudoginsenosides. Ginsenosides and pseudoginsenosides are active ingredients of the ginseng root (Blumenthal, 2001) that have been shown to stimulate Ca^{2+} -activated Cl^- channels by activation of phospholipase C and mobilization of intracellular Ca^{2+} (Choi et al., 2001). Moreover, ginsenoside Re has been shown to increase NO, which activates K^+ and Ca^{2+} channels as well as Cl^- secretion via wt-CFTR and mutant Phe508del-CFTR (Dong et al., 1995; Kamosinska et al., 1997; Bai et al., 2004; Lee et al., 2004). Taken together, the use of natural plant products provides new avenues for the treatment of CF. *P. acidus* extract can thus be used to enhance the activity of CFTR mutants with residual function or, in combination with compounds that rescue mutants with traffic defects such as Phe508del-CFTR, to further stimulate the Cl^- channel activity of these mutants.

Acknowledgments

The excellent assistance by Ernestine Tartler and Agnes Paech is gratefully acknowledged. A.R. and A.S. are recipients of postdoctoral and PhD fellowships SFRH/BD/19415/2004 and SFRH/BPD/14653/2004, respectively (FCT, Portugal). We thank L. A. Clarke (Department of Chemistry and Biochemistry, University of Lisboa, Portugal) for reading and correcting the manuscript.

References

- Bai CX, Takahashi K, Masumiya H, Sawanobori T, and Furukawa T (2004) Nitric oxide-dependent modulation of the delayed rectifier K^+ current and the L-type Ca^{2+} current by ginsenoside Re, an ingredient of Panax ginseng, in guinea-pig cardiomyocytes. *Br J Pharmacol* **142**:567–575.
- Berger AL, Randak CO, Ostedgaard LS, Karp PH, Vermeer DW, and Welsh MJ (2004) Curcumin stimulates CFTR Cl^- channel activity. *J Biol Chem* **280**:5221–5226.
- Bjarnsholt T, Jensen PO, Rasmussen TB, Christophersen L, Calum H, Hentzer M, Hougen HP, Rygaard J, Moser C, Eberl L, et al. (2005) Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. *Microbiology* **151**:3873–3880.
- Blumenthal M (2001) Asian ginseng: potential therapeutic uses. *Adv Nurse Pract* **9**:26–28.
- Bobadilla JL, Macek M Jr, Fine JP, and Farrell PM (2002) Cystic fibrosis: a worldwide analysis of CFTR mutations—correlation with incidence data and application to screening. *Hum Mutat* **19**:575–606.
- Boucher RC (2004) New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur Respir J* **23**:146–158.
- Cermak R, Follmer U, and Wolfram S (1998) Dietary flavonol quercetin induces chloride secretion in rat colon. *Am J Physiol* **275**:G1166–G1172.
- Choi S, Rho SH, Jung SY, Kim SC, Park CS, and Nah SY (2001) A novel activation of Ca^{2+} -activated Cl^- channel in *Xenopus* oocytes by ginseng saponins: evidence for the involvement of phospholipase C and intracellular Ca^{2+} mobilization. *Br J Pharmacol* **132**:641–648.
- Clancy JP, Ruiz FE, and Sorscher EJ (1999) Adenosine and its nucleotides activate wild-type and R117H CFTR through an A2B receptor-coupled pathway. *Am J Physiol* **276**:C361–C369.
- Cohen BE, Lee G, Jacobson KA, Kim YC, Huang Z, Sorscher EJ, and Pollard HB (1997) 8-Cyclopentyl-1,3-dipropylxanthine and other xanthines differentially bind to the wild-type and deltaF508 mutant first nucleotide binding fold (NBF-1) domains of the cystic fibrosis transmembrane conductance regulator. *Biochemistry* **36**:6455–6461.
- deCarvalho AV, Ndi CP, Tsopmo A, Tane P, Ayafor J, Connolly JD, and Teem JL (2002) A novel natural product compound enhances camp-regulated chloride conductance of cells expressing CFTR-f508del. *Mol Med* **8**:75–87.
- Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, and Welsh MJ (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature (Lond)* **358**:761–764.
- Dong YJ, Chao AC, Kouyama K, Hsu YP, Bocian RC, Moss RB, and Gardner P (1995) Activation of CFTR chloride current by nitric oxide in human T lymphocytes. *EMBO (Eur Mol Biol Organ) J* **14**:2700–2707.
- Egan ME, Pearson M, Weiner SA, Rajendran V, Rubin D, Glockner-Pagel J, Canny S, Du K, Lukacs GL, and Caplan MJ (2004) Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science (Wash DC)* **304**:600–602.
- Farinha CM and Amaral MD (2005) Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. *Mol Cell Biol* **25**:5242–5252.
- Fischer H, Schwarzer C, and Illek B (2004) Vitamin C controls the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc Natl Acad Sci USA* **101**:3691–3696.
- Huang P, Lazarowski ER, Tarran R, Milgram SL, Boucher RC, and Stutts MJ (2001) Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells. *Proc Natl Acad Sci USA* **98**:14120–14125.
- Hwang T-C, Wang F, Yang IC, and Reenstra WW (1997) Genistein potentiates wild-type and delta F508-CFTR channel activity. *Am J Physiol* **273**:C988–C998.
- Illek B and Fischer H (1998) Flavonoids stimulate Cl^- conductance of human airway epithelium in vitro and in vivo. *Am J Physiol* **275**:L902–L910.
- Jansakul C, Intarit K, Itharat A, Phadungcharoen T, Ruangrunsi N, Merica A, and Lange GL (1999) Biological activity of crude extract and saponin pseudoginsenoside-RT1 derived from the fruit of *Randia siamensis*. *Pharmaceutical Biology* **37**:42–45.
- Kamosinska B, RADOMSKI MW, Duszyk M, Radomski A, and Man SF (1997) Nitric oxide activates chloride currents in human lung epithelial cells. *Am J Physiol* **272**:L1098–L1104.
- Kanchanapoom T, Kasai R, and Yamasaki K (2001) Acetylated triterpene saponins from the Thai medicinal plant, *Sapindus emarginatus*. *Chem Pharm Bull (Tokyo)* **49**:1195–1197.
- Kerem E (2005) Pharmacological induction of CFTR function in patients with cystic fibrosis: mutation-specific therapy. *Pediatr Pulmonol* **40**:183–196.
- Kunzelmann K and Mall M (2003) Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. *Am J Respir Med* **2**:299–309.
- Lee BH, Jeong SM, Ha TS, Park CS, Lee JH, Kim JH, Kim DH, Han JS, Kim HC, Ko SR, et al. (2004) Ginsenosides regulate ligand-gated ion channels from the outside. *Mol Cells* **18**:115–121.
- Li H and Wang Q (2004) Evaluation of free hydroxyl radical scavenging activities of some Chinese herbs by capillary zone electrophoresis with amperometric detection. *Anal Bioanal Chem* **378**:1801–1805.
- Lim M, McKenzie K, Floyd AD, Kwon E, and Zeitlin PL (2004) Modulation of DeltaF508 CFTR trafficking and function with 4-PBA and flavonoids. *Am J Respir Cell Mol Biol* **31**:351–357.
- Lin MW, Yang SR, Huang MH, and Wu SN (2004) Stimulatory actions of caffeic acid phenethyl ester, a known inhibitor of NF- κ B activation, on Ca^{2+} -activated K^+ current in pituitary GH3 cells. *J Biol Chem* **279**:26885–26892.
- Ma T, Vetrivel L, Yang H, Pedemonte N, Zegarar-Moran O, Galiotta LJ, and Verkman AS (2002) High-affinity activators of cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance identified by high-throughput screening. *J Biol Chem* **277**:37235–37241.
- Mall M, Wissner A, Hübner M, Kühr J, Brandis M, Greger R, and Kunzelmann K (2000) Effect of genistein on native epithelial tissues from normal individuals and CF patients and on CFTR expressed in *Xenopus* oocytes. *Br J Pharmacol* **130**:1884–1892.
- Montero M, Lobaton CD, Hernandez-Sanmiguel E, Santodomingo J, Vay L, Moreno A, and Alvarez J (2004) Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. *Biochem J* **384**:19–24.
- Moran O and Zegarar-Moran O (2005) A quantitative description of the activation and inhibition of CFTR by potentiators: genistein. *FEBS Lett* **579**:3979–3983.
- Mueller-Oerlinghausen B, Ngamwathana W, and Kanchanape P (1971) Investigation into Thai medicinal plants said to cure diabetes. *J Med Assoc Thai* **54**:105–112.
- Panthong A, Kanjanapothi D, and Taylor WC (1986) Ethnobotanical review of medicinal plants from Thai traditional books, part I: plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. *J Ethnopharmacol* **18**:213–228.
- Pedemonte N, Lukacs GL, Du K, Caci E, Zegarar-Moran O, Galiotta LJ, and Verkman AS (2005) Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J Clin Invest* **115**:2564–2571.
- Song Y, Sonawane ND, Salinas D, Qian L, Pedemonte N, Galiotta LJ, and Verkman AS (2004) Evidence against rescue of defective Δ F508-CFTR cellular processing by curcumin in cell culture and mouse models. *J Biol Chem* **279**:40629–40633.
- Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, and Boucher RC (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science (Wash DC)* **269**:847–850.
- Suaud L, Carattino M, Kleyman TR, and Rubenstein RC (2002) Genistein improves regulatory interactions between G551D-cystic fibrosis transmembrane conduc-

tance regulator and the epithelial sodium channel in *Xenopus* oocytes. *J Biol Chem* **277**:50341–50347.

Suaud L, Li J, Jiang Q, Rubenstein RC, and Kleyman TR (2001) Genistein restores functional interactions between F508del-CFTR and ENaC in *Xenopus* oocytes. *J Biol Chem* **277**:8928–8933.

Van Goor F, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings LR, Miller M, et al. (1996) RESCUE OF F508del-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small

molecules. *Am J Physiol* **290**:L1117–L1130.

Wolfender JL, Ndjoko K, and Hostettmann K (2001) The potential of LC-NMR in phytochemical analysis. *Phytochem Anal* **12**:2–22.

Address correspondence to: Prof. Dr. Karl Kunzelmann, Institut für Physiologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany. E-mail: uqkkunze@mailbox.uq.edu.au
