Regulation of volume-sensitive chloride channels by cystic fibrosis transmembrane conductance regulator and epidermal growth factor receptor

Iskandar F. Abdullaev

Department of Physiological Sciences

School of Life Science

The Graduate University for Advanced Studies

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ABBREVIATION LIST

AE – Anion Exchanger (Cl^-/HCO_3^- antiporter)

AQP – aquaporin

BPV – Bovine papillomavirus

CaCC – Calcium-activated chloride channel

CF – cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

DRA – down-regulated in adenoma

EBP50 – Ezrin-binding phosphoprotein 50

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

ENaC – Epithelial sodium channel

GFP – Green fluorescence protein

IK – intermediate-conductance K^+ channel

IsK – slow K^+ channel

MDR –Multidrug-resistance protein

NBD – Nucleotide binding domain

NHE – Na^+/H^+ exchanger (Na^+/H^+ antiporter)

NHE-RF – Na^+/H^+ exchanger regulatory factor

NKCC – Na^+-K^+-$2Cl^-$ cotransporter

ORCC – Outwardly rectifying chloride channel

PGP – P-glycoprotein

PDGF – Platelet-derived growth factor

PDZ – Postsynaptic density-95/Dlg-A/Zonula Occludens-1

R-domain – regulatory domain

ROMK – Renal outer medulla K channel

RVD – Regulatory volume decrease

TM – Transmembrane domain

VSOR – Volume-sensitive outwardly rectifying

WT – Wild type

ABSTRACT

Effectene-mediated transient expression of wild-type (WT) human cystic fibrosis transmembrane conductance regulator (CFTR) in HEK293T cells resulted in a 3-times decrease of the amplitude of steady-state volume-sensitive outwardly rectifying (VSOR) chloride current. Expression of the CFTR ΔF508 mutant, which cannot be translocated to the plasma membrane, failed to mimic the CFTR effect on the VSOR Cl⁻ current, suggesting that plasma membrane expression of CFTR protein is necessary for its regulatory effect on the VSOR Cl⁻ channel. Expression of the G1349D mutant of CFTR, which impairs ATP binding to the NBD2 domain of CFTR protein, failed to inhibit VSOR Cl⁻ currents. D1370N and K1250M mutations in NBD2 domain, which impair ATP hydrolysis, were also ineffective in down-regulating VSOR Cl⁻ currents. In contrast, expression of G551D mutant, which impairs ATP binding to the NBD1 domain, mimicked the effect of WT-CFTR. Thus, I conclude that plasma membrane expression of an ATP-hydrolysable conformation of the NBD2 domain of CFTR is essential for its down-regulatory action on the volume-sensitive chloride conductance in HEK293T/CFTR cells.

In mouse mammary gland C127 cells, in contrast, VSOR Cl⁻ currents were up-regulated by stable expression of CFTR mediated by bovine papillomavirus (BPV), a carrier for CFTR gene transfection, by around 3-fold. Also, BPV-mediated expression with CFTR ΔF508 mutant produced a similar up-regulating effect on VSOR Cl⁻ currents. BPV expression is known to constitutively activate receptors to platelet-derived growth factor (PDGF) and epidermal growth factor

(EGF). Application of a PDGF peptide or a specific inhibitor of PDGF tyrosine kinase, tyrphostin AG1296, had no effect on VSOR Cl⁻ currents. In contrast, application of an EGF peptide enhanced VSOR Cl⁻ currents in C127 cells, but not in C127 cells treated with BPV (C127/BPV cells). A specific inhibitor of EGF-receptor tyrosine kinase, tyrphostin B46, profoundly suppressed VSOR Cl⁻ currents in C127 and C127/BPV cells. Thus, I conclude that VSOR Cl⁻ channels are enhanced by an EGF receptor tyrosine kinase signaling and that BPV-induced up-regulation overrides CFTR-induced down-regulation of VSOR Cl⁻ channels in C127/CFTR cells. Since inhibitors of phospholipase Cγ (PLCγ), phosphatidylinositol-3-kinase (PI3K) and MAP kinase kinase (MEK) failed to affect the VSOR Cl⁻ channel activity, it might be possible that the VSOR Cl⁻ channel is regulated by some signaling pathway, other than those involving PLCγ, PI3K and MEK, which is downstream to the EGF receptor tyrosine kinase.

INTRODUCTION

Virtually all cells go through osmotic transitions during lifetime, since both intracellular metabolism and membrane transport are expected to produce fluctuations in concentrations of osmotically active constituents. Cellular swelling in response to a hypo-osmotic challenge activates anion channels in most cell types (Strange et al., 1996; Nilius et al., 1997; Okada, 1997). Parallel activation of potassium channels allows simultaneous exits of K^+ and Cl^- ions via both electroconductive pathways. Obligatory water leaves the cells thereby restoring their original volume, a process termed the regulatory volume decrease (RVD). Volume-sensitive outwardly rectifying (VSOR) chloride channels are expressed to different degrees in a variety of cell types. Cell volume regulation in response to a hypotonic challenge has first been recognized as a primary function for this type of channel (Strange et al., 1996; Nilius et al., 1997; Okada, 1997). However, numerous recent studies have evolved around several other physiological processes, where VSOR Cl^- channels are key players. These include intracellular acid-base balance due to permeability to lactate and bicarbonate ions (Nilius et al., 1998), cell proliferation (Voets et al., 1995, 1997), cell cycle (Shen et al., 2000; Wondergem et al., 2001) and apoptosis (Maeno et al., 2000; Okada et al., 2001). In most cells, osmotic swelling activates macroscopic Cl^- currents with moderate outward rectification and inactivation kinetics at large positive potentials in the conventional whole-cell mode. The VSOR anion channels feature a low-field anion selectivity with a permeability sequence of $SCN^- > I^- > Br^- > Cl^- > F^- >$ gluconate$^-$ in a human intestinal cell line (Okada, 1997). Single VSOR Cl^- channels exhibit an intermediate unitary conductance of $50-70$ pS and sensitivity to extracellular ATP (Tsumura et al., 1996). The VSOR Cl^- channel activity

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requires intracellular ATP; a prerequisite for channel activation is not energy of ATP hydrolysis, but direct binding of ATP molecules to the channel protein or its accessory protein (Okada, 1997).

Conventional chloride channel blockers, such as SITS, NPPB and DPC, inhibit the swelling-activated chloride current with different efficiency. Although no specific blocker of VSOR Cl$^-$ channel has been found so far, some chemicals belonging to different classes of modulators have been shown to affect the VSOR Cl$^-$ channel activity (for review see Okada, 1997; Nilius et al., 1997). Unexpectedly, P-glycoprotein (PGP) inhibitors, such as verapamil, nifedipine, 1,9-dideoxiforskolin (DDFSK), tamoxifen and quinidine, were found to suppress VSOR Cl$^-$ currents in multidrug-resitance protein (MDR1)-transfected cells (Valverde et al., 1992; Mintenig et al., 1993). This was a basis for a *transporter/channel bifunctional* hypothesis (Valverde et al., 1992; Gill et al., 1992; Mintenig et al., 1993) which suggests that PGP can function as a transporter for active pumping out of the drugs in normal conditions, whereas cellular swelling would turn this protein into a channel for conducting chloride ions. However, following studies have shown that VSOR Cl$^-$ channel functions independently of PGP expression, though the MDR1 protein can modulate this channel (Tominaga et al., 1995; Miwa et al, 1997; see also Okada, 1997).

P-glycoprotein is a member of ATP-binding cassette (ABC) transporter gene family. Cystic fibrosis transmembrane conductance regulator (CFTR) also belongs to ABC transporter proteins and shares great structural similarity with PGP. Some mutations in this gene cause cystic fibrosis, an autosomal recessive disease, characterized by severe dysfunction of fluid and electrolyte transport in secretory epithelia. In contrast to PGP, CFTR has been shown to actually function as a chloride channel (for review see Akabas, 2000), although with properties quite distinct from the VSOR Cl$^-$ channel: a small-conductance (8 − 10 pS)

nonrectifying channels activated by PKA-mediated phosphorylation. Recent studies showed that the CFTR protein is not only a Cl^- channel but also acts as a regulator of other channels and transporters (reviewed by Kunzelmann and Schreiber, 1999; Schwiebert et al., 1999): 1) Co-expression with CFTR conferred glibenclamide sensitivity to the inward rectifying potassium channel, ROMK1 (Ruknudin et al., 1998), ROMK2 (McNicholas et al., 1996, 1997) and Kir6.1 (Ishida-Takahashi et al., 1998); 2) the epithelial sodium conductance was also found to be controlled by CFTR by inhibiting an amiloride-sensitive epithelial Na^+ channel, ENaC (Stutts et al., 1995, 1997; Schreiber et al., 1999); 3) an outwardly rectifying chloride channel, ORCC, which have been shown to be highly expressed in some epithelial cells, was shown to be tightly controlled by CFTR (Egan et al., 1992; Gabriel et al., 1993; Jovov et al., 1995a, b; Fulmer et al., 1995; Schwiebert et al., 1998); 4) Ca^{2+}-activated chloride channels (CaCC), that play a key role in chloride transport in a variety of cell types, were found to be down-regulated by CFTR expression in *Xenopus* oocytes (Kunzelmann et al., 1997) and a CPAE endothelial cell line (Wei et al., 1999, 2001); 5) CFTR was shown to up-regulate the Cl^-/HCO_3^- exchange, AE (Lee et al., 1999a,b; Wheat et al., 2000; Choi et al., 2001), supposedly by augmenting the expression level of a putative anion exchanger protein, DRA ("down-regulated in adenoma"), but not a ubiquitous basolateral anion exchanger isoform, AE2 (Wheat et al., 2000); 6) increased expression of the NHE3 isoform of electroneutral Na^+/H^+ exchangers was recently found to mediate the impaired bicarbonate salvage mechanism in cystic fibrosis (Ahn et al., 2001); 7) CFTR also up-regulated the expression of $Na^+-K^+-2Cl^-$ cotransporter, NKCC1, in pancreatic duct cells (Shumaker and Soleimani, 1999); and 8) CFTR activated an aquaporin family member, AQP3, in airway epithelial cells (Schreiber et al., 1999, 2000).

Thus, CFTR, a unique multifunctional protein, is a key regulator of a multitude of ion-transporting systems crucial for electrolyte balance and secretion. Is the cell

volume-regulatory machinery in general or and a volume-sensitive chloride channel in particular a subject for control by CFTR? Indeed, loss of CFTR protein expression in CFTR knockout mice (Valverde et al., 1995, 2000; Lock and Valverde, 2000) or in the plasma membrane of human cystic fibrosis tracheal cells (Vazquez et al., 2001) led to the impaired regulatory volume decrease. These authors suggested that swelling-activated potassium channels composed of the KvLQT/Isk complex in murine cells (Lock and Valverde, 2000) and hIK channels in human CF cells (Vazquez et al., 2001) are primarily impaired in cystic fibrosis, although they have not investigated the VSOR Cl⁻ channel activity in these model systems. Volume-sensitive Cl⁻ currents in guinea pig atrial myocytes (Sakaguchi et al., 1997), in human intestinal epithelial cells (Liu et al., 1998) and in rat pancreatic β-cells (Best and Benington, 1998) were found to be sensitive to the sulfonylurea glibenclamide, a well known antagonist of CFTR. This could be, at least in part, due to direct or indirect regulation of VSOR Cl⁻ channel by this ABC transporter. Consistently, Chan et al. (1992) reported that the antibody raised against R-domain of CFTR did not affect the Ca^{2+}-activated Cl⁻ currents, but significantly attenuated the swelling-activated whole-cell Cl⁻ currents in human colonic epithelial T84 cells. More direct evidence for regulation of VSOR Cl⁻ channels by CFTR was provided by Vennekens et al. (1999): Heterologous expression of CFTR protein in CPAE endothelial cells and COS cells resulted in significant down-regulation of volume-regulated chloride currents.

Obviously, control by CFTR of the cell volume regulation and volume-sensitive chloride conductance is of great physiological importance for the tissues and organs where these two proteins are expressed, including heart, gastrointestinal tract, lung and kidney. However, the mechanism of regulation of RVD and VSOR chloride channels by CFTR is poorly understood at present. Therefore, in this study, I first aimed to determine, by employing a site-directed mutagenesis

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technique, the structural determinant of CFTR-induced down-regulation of VSOR Cl⁻ currents in HEK293T cells transiently transfected with CFTR.

In murine mammary C127 cells, in contrast, I found an apparent CFTR-insensitivity of VSOR Cl⁻ channel activity, when stably transfected with CFTR using bovine papilloma virus (BPV). Moreover, I found rather an up-regulating effect. Since expression of some BPV proteins results in constitutive activation of PDGF- and EGF-receptors, in the present study, I second aimed to investigate a possible regulation of VSOR Cl⁻ channel by the PDGF-receptor or EGF-receptor tyrosine kinase pathway.

MATERIALS AND METHODS

Cells

A murine mammary adenocarcinoma cell line, C127, was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). C127 cells that had been stably transfected by BPV either without (C127/Mock) or with the ΔF508 mutant of CFTR (C127/ΔF508), were provided by Dr. H. Cheng (Genzyme Corporation, Framingham, MA). C127 cells stably expressing CFTR via BPV (C127/CFTR) were kindly provided by Dr. M. J. Welsh (University of Iowa), and cultured in DMEM with 10% FCS and 200 μg ml^{-1} geneticin. Human embryonic kidney HEK293T cells were grown in DMEM with 10% FCS and used in experiments before and after transient transfection of CFTR.

Cells attached on the plastic substrate were re-suspended by mechanical detachment, as reported previously (Kubo and Okada, 1992), and cultured with agitation for 15-300 min. Then, cells were placed in a chamber and washed by bathing solution after attaching them to the glass bottom of the chamber. A hypotonic challenge was made by switching the perfusate from isotonic to hypotonic solution.

Chemicals

All the agents except for Na-HEPES (Nacalai Tesque, Kyoto, Japan), forskolin and EGTA (Wako, Osaka, Japan) were obtained from Calbiochem-Novabiochem Corporation (San Diego, CA). The stock solutions were prepared by dissolving in dimethyl sulfoxide (except growth factors, see below), stored at −20°C and diluted 1000 times in solutions just before use.

PDGF was reconstituted in 1 ml of sterile 4 mM HCl solution containing 0.1% bovine serum albumin and stored at −20°C. EGF was dissolved in 10 mM HEPES buffer (pH 7.4). All vehicles did not affect the VSOR Cl⁻ current at the used concentration (less than 0.1%).

SDS-PAGE and immunoblotting

HEK293T cells were treated with SDS-PAGE sample buffer and frozen. Samples were warmed at 37°C for 10 min and sonicated 1 s before analysing on 7.5% SDS-polyacrylamide gels (Laemmli, 1970). The proteins on the gel were electrophoretically transferred onto nitrocellulose membranes and incubated with the primary antibody raised against the intracellular R-domain of human CFTR (R&D Systems, Inc., Minneapolis, MN) as well as actin as an internal control. The reaction was visualized with biotinylated second antibodies and streptavidin-conjugated alkaline phosphatase (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (SIGMA, St. Louis, MO) as substrates for detection of alkaline phophatase. The visualized signals corresponding to CFTR, CFTR mutants and actin proteins were scanned to analyze their intensity.

Molecular biology

We generated point mutations by a standard PCR-based cloning strategy using pBQ6.2 (a kind gift from Dr. J. R. Riordan, S.C. Medical Research Center, Arizona) which contains a full-length CFTR cDNA insert. After confirming the identities of individual clones through double-stranded plasmid sequencing (ABI310, Perkin Elmer Inc., Wellesley, MA), the coding regions of both wild-type and mutated CFTR cDNA were subcloned into pCINeo-IRES-GFP (a kind gift from Dr. J. Eggermont, KUL) to produce expression vectors.

HEK293T cells were transiently transfected with Effectene (Qiagen, Valencia, CA). The day before transfection cells were seeded on 6-well plates to get 40-80%

confluency (usually about 50%, estimated visually) on the day of transfection. Transfection was performed according to the manufacture's instructions. In brief, DNA was dissolved in the DNA-condensation buffer. Enhancer was added and mixed by vortexing. Then the mixture was incubated at room temperature, and Effectene Transfection Reagent was added. Complex formation was allowed by incubation at room temperature. While complex formation took place, cells were washed by PBS, and fresh DMEM containing 10% FCS was added to the cells. DMEM containing 10% FCS was added to the reaction tube containing the transfection complexes, mixed by pipetting and immediately drop-wised onto the cells. Electrophysiological experiments were done during 24-48 h after transfection. Since incorporation of wild-type (WT) or mutant CFTR in the vector allows expression of both channel and green fluorescent protein (GFP), GFP-positive cells, which could be detected in patch-clamp setup directly under a fluorescence microscope, should express CFTR.

RNA isolation and RT-PCR

Poly (A)$^+$ RNA was extracted from C127 cells using Direct mRNA Purification Kit with magnetic porous glass (MPG) (CPG Inc., New Jersey USA). Briefly, the cells were homogenized in extraction-hybridization buffer, which contained 100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulphate (LiDS) and 5 mM dithiothreitol (DTT). Poly (A)$^+$ RNA was magnetically isolated from the homogenate by binding with MPG-bound oligo(dT)$_{25}$. The poly (A)$^+$ RNA of 200 ng was used for single-strand cDNA synthesis using SuperScript Pre-amplification System (Life technologies, Rockville, USA). In brief, RNA samples were reverse transcribed at 42°C for 50 min by incubation with 20 µl of an RT mixture containing the following constituents: 500 ng of oligo(dT)$_{12-18}$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl$_2$, 1mM dNTPs, 10 mM DTT and 200 units of SuperScript II RT. The reverse transcriptase was then inactivated by

heating at 70°C for 15 min. The resultant first-strand cDNA was used for the PCR procedure. PCR was performed in a total volume of 50 µl of a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM NTPs, and 250 units of Ampli Taq Gold (Perkin Elmer, New Jersey, USA), using the following set of primers (200 nM each), which were based on the sequence of the mouse EGFR. The sense and antisense primers were 5'-GAACACAAGGACAACATTGGC-3' and 3'-TCCATCATAAGGCTTGGACC-5', respectively. The set of primers is predicted to yield a 320-bp product. The optimum temperature cycling protocol was determined to be 94°C for 1 min, 50°C for 1 min and 72°C for 2 min for 1 round of 35 (or 25) cycles using a programmable thermal cycler (GeneAmp PCR System 9600, Perkin Elmer). Two sets of negative control experiments were performed by including primers but no cDNA or RNA that was not reverse-transcribed. G3PDH, a house-keeping protein with abundant expression, was used as a positive control. PCR products were directly subjected to sequencing using an ABI PRISM 310 automatic sequencer (Perkin-Elmer).

Confocal immunofluorescence microscopy

HEK293T cells cultured on coverslips had been transiently transfected with the CFTR protein or the mutant were fixed with 1% formaldehyde in PBS for 15 min. After brief washing with PBS, they were permeabilized with PBS containing 0.2% Triton X-100 for 15 min. Then they were washed three times with PBS and soaked in a blocking solution (PBS-containing 1% BSA) for 10 min. Coverslips were then incubated with the monoclonal anti-CFTR (R-domain specific) mouse antibody (Genzyme) for 1 h and washed three times with PBS. After that, they were incubated with the rhodamine-conjugated goat anti-mouse IgG (CHEMICON International, Temecula, CA) for 30 min, followed by washing with PBS. Samples were examined using Zeiss laser scanning microscope LSM-510 (Carl Zeiss Jena GmbH, Jena, Germany).

Electrophysiology

Whole-cell recordings were performed, as reported previously (Kubo and Okada, 1992; Liu et al., 1998). The patch electrodes were fabricated from borosilicate glass capillaries using a micropipette puller (Sutter Instrument, Novato, CA). Electrodes, filled with pipette solution, had a resistance of about 2 MΩ. Series resistance (usually < 5 MΩ) was compensated (70-80%) to minimize voltage errors. Currents were recorded using Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz using a four-pore Bessel filter and digitized at 4 kHz.

The PCLAMP software (version 6.0.2, Axon Instruments) was used for command pulse control, data acquisition and analysis. The time course of current activation and recovery was monitored by repetitively (every 15 s) applying alternating step pulses from a holding potential of 0 mV to ±40 mV. To observe voltage dependence of the current (inactivation kinetics at large positive potentials), stepping pulses were applied from a pre-potential at −100 mV to test potentials of −100 to +100 mV in 20 mV increments after reaching steady-state level of swelling-activated current. The amplitude of instantaneous current was measured at 1.25 ms after the step pulse onset.

The isotonic bath solution contained (mM): 110 CsCl, 2 CaCl$_2$, 1 MgCl$_2$, HEPES 5, glucose 5, mannitol 50. pH was adjusted by CsOH to 7.4. Osmolality of this solution was 275±3 mOsmol-kg H$_2$O^{-1}. The hypotonic bath solution was obtained by removal of mannitol, and its osmolality was 230±3 mOsmol-kg H$_2$O^{-1}. The pipette solution contained (mM): CsCl 110, MgSO$_4$ 2, Na$_2$ATP 1, EGTA 1, NaHEPES 15, HEPES 10 (pH 7.3, 250 ±3 mOsmol-kg H$_2$O^{-1}).

Single-channel recordings were carried out in the cell-attached mode, as described previously (Okada et al., 1994). Gigaseal was formed by pipette attachment to the cell, pre-swollen in hypotonic high K$^+$ solution containing (mM): KCl 100, CaCl$_2$ 2, MgCl$_2$ 1, glucose 5, HEPES 5 (pH adjusted to 7.4 with KOH). Osmolality was

215 ± 3 mOsmol-kg H_2O^{-1}. Pipette solution was identical to the hypotonic bath solution used for whole-cell experiments.

Intracellular pH measurements

Intracellular pH (pH_i) was measured by pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxy methyl ester (BCECF/AM). The fluorescence from a single cell was monitored by SIT camera with an emission wavelength of 510 nm and excitation wavelengths of 490 and 450 nm. A calibration curve was generated using the high KCl-nigericin technique and solutions of varying pH (6.6, 7.0, 7.4 and 7.8) as reported previously (Ritucci et al., 1996). The fluorescence ratio at 490/450 nm was plotted against pH_i and fitted by a linear regression.

Volume measurements and osmotic water permeability evaluation

The mean cell volume for RVD experiments was measured at room temperature using a Coulter Counter (CDA-500, Sysmex, Kobe, Japan), as reported previously (Hazama and Okada, 1988).

Volume measurements in the patch-clamp whole-cell mode were performed, as described previously (Morishima et al., 1998). Briefly, the automatic edge determination system composing of a CCD camera, an image capture adapter and a personal computer were used. Cell images were recorded every 5 s for further analysis.

An osmotic water permeability, P_f, was calculated, as reported previously (Sabirov et al., 1998), using the following equation:

$$P_f = [V_o/(SV_w\Delta\pi)]/[(dV/V_o)/dt]$$

where   S – surface area (cm^2)

   V_w – the partial molar volume of water (18 cm^3/mol)

   $\Delta\pi$ – the osmotic gradient

$(dV/V_0)/dt$ – the rate of relative volume change.

Data analysis

Data analysis was performed using WinASCD (kindly provided by G. Droogmans, KUL), CLAMPFIT (Axon Instruments) and Puls+Pulsfit (Heka Elektronik, Lambrecht, Germany) software packages. Statistical analysis, fittings and graphs were done in Origin 6.0 or 6.1 (OriginLab Corporation, Northampton, MA). To eliminate variations caused by different cell size, we have normalized current amplitudes by unit membrane capacitance. Mean values are given by ± s.e.m. from n cells. Statistical significance was tested by Student's paired t-test using $p < 0.05$ as the level of significance.

RESULTS

Effect of CFTR expression on VSOR chloride conductance in human embryonic kidney HEK293T cells

Down-regulation of VSOR Cl^- currents by CFTR in HEK293T cells

The elevation of intracellular cAMP by a forskolin/dbcAMP cocktail did not activate any whole-cell currents in non-transfected control human epithelial kidney (HEK293T) cells ($n=3$, data not shown) indicating lack of functional expression of endogenous CFTR channel. Lack of molecular expression of CFTR protein in HEK293T cells was confirmed by indirect immunofluorescence assay using anti-CFTR antibody raised against the intracellular R-domain, as shown in Fig. 1A.

After transient transfection of HEK293T cells with WT-CFTR, in contrast, molecular expression of CFTR was detected on the plasma membrane by immunostaining (Fig. 1B: WT). Stimulation with a forskolin/dbcAMP cocktail resulted in activation of large whole-cell current (Fig. 2A). The current responses to step pulses exhibited little time-dependent activation and inactivation (Fig. 2B). The current-voltage curve exhibited a linear relationship (Fig. 2C). These characteristics are typical of CFTR Cl^- current. In contrast, cAMP-cocktail stimulation failed to evoke any currents in ΔF508 mutant-expressing cells (Fig. 2).

As shown in Fig. 3, in both Mock (CFTR-lacking) and CFTR-expressing cells, a hypotonic challenge induced activation of ionic currents with electrophysiological properties (outward rectification and voltage-dependent inactivation at large positive potentials) typical of volume-sensitive outwardly rectifying (VSOR) Cl^-

currents, which have been characterized in details in many different cell types (Strange et al., 1996; Nilius et al., 1997; Okada, 1997). Although overall properties of VSOR currents were similar in both cell preparations, the peak current amplitude was significantly lower in CFTR-expressing cells compared to the control cells (Fig. 3). This result is in agreement with the previous observation of VSOR Cl⁻ current inhibition by CFTR in COS cells (Vennekens et al., 1999).

Molecular determinants of VSOR regulation by CFTR

To clarify the molecular basis for CFTR-VSOR interaction, we introduced several mutations into the CFTR gene. Figure 4 illustrates our basic strategy. Deletion of phenylalanine at the position 508 in the first NBD (mutant **ΔF508**) is most frequently occurring mutation which accounts for about 70% of cystic fibrosis cases in Caucasians and results in impaired membrane trafficking of CFTR protein (Denning et al., 1992). Deletion of last three amino acids TRL (mutant **ΔTRL**) prevents CFTR binding to a number of proteins containing PDZ-domains, such as EBP50 or NHERF (Short et al., 1998). **G551D** and **G1349D** are naturally occurring CF mutations in the NBD1 and NBD2 domains, respectively. These two mutations are known to decrease the nucleotide binding at NBD1 and NBD2. G551D mutation has been shown to impair CFTR-induced regulation of the outwardly rectifying Cl⁻ channel, ORCC (Fulmer et al., 1995; Jovov et al., 1995a, b) and the epithelial Na⁺ channel, ENaC (Kunzelmann et al., 2000).

Mutation K1250 is located in consensus Walker motif A of the putative ATP-binding pocket in NBD2 and interacts directly with β- and γ-phosphate groups of bound ATP. The D1370 mutation is in the Walker consensus B motif and contributes to coordination of Mg^{2+}. Mutations **K1250M** and **D1370N** impair the ATP hydrolysis at NBD2 perhaps without changing the ATP binding (Gunderson and Kopito, 1995; Wilkinson et al., 1996). The (K1250A and D1370N) mutants

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greatly prolonged the burst activity and induced a slight decrease in the inter-burst interval (Carson et al., 1995). A minor increase and no change were induced by K1250A and D1370N, respectively, in over-all P_o (Gunderson and Kopito, 1995).

Plasma membrane expression of all used mutants other than ΔF508 was confirmed by western blotting (Fig. 5).

Figure 6 shows immunofluorescence images of HEK293T cells expressing mutant CFTR proteins. For the ΔF508 mutant, the fluorescence pattern was not clearly observed on the plasma membrane (top, left), but was found to be homogeneous within the cell except for the nucleus region (top, right). For mutants of G551D (top, right), G1349D (middle, left) and ΔTRL (middle, right), in contrast, fluorescence was sharpest on the plasma membrane, whereas that became dim when focused on the middle part of the cells (not shown). Similar fluorescence patterns were also observed in HEK293T cells expression K1250M and D1370N mutants (bottom panels). In the light of these results, it can be concluded that CFTR mutants of G551D, G1349D, ΔTRL, K1250M and D1370N, but not ΔF508, were expressed on the plasma membrane in HEK293T cells.

First, I examined if the VSOR-inhibiting effect of CFTR was actually related to its plasma membrane localization. Deletion of phenylalanine at the position 508 (ΔF508) completely abolished the effect of CFTR (data not shown). The VSOR current densities in ΔF508-expressing cells were indistinguishable from those measured in control (Mock) cells. VSOR Cl⁻ current densities in ΔF508 mutant-expressing cells were not statistically different from those in Mock-expressing cells (data not shown). This result suggests that plasmalemmal localization is critical for CFTR-induced inhibition of volume-sensitive Cl⁻ channel.

Next, I addressed the question as to whether CFTR-induced regulation is mediated by protein-protein interaction at the C-terminal domain. The ΔTRL mutant

inhibited VSOR Cl⁻ currents as effectively as WT-CFTR (Fig. 7). Therefore, it appears that the C-terminus-mediated binding to the putative PDZ-domains on VSOR channel molecule is not involved in its regulation by CFTR.

The involvement of the nucleotide-binding domain NBD1 or NBD2 was tested by introducing G551D and G1349D mutants, which are naturally occurring CF mutations, and known to decrease nucleotide binding to NBD1 and NBD2, respectively (Carson et al., 1995). The G551D mutant inhibited VSOR chloride currents to the same extent as WT-CFTR (Fig. 7), suggesting that ATP binding to the NBD1 domain is not essential for CFTR-induced VSOR inhibition. On the other hand, the G1349D, K1250M and D1370N mutants failed to significantly affect VSOR Cl⁻ currents (Fig. 7), although they were expressed on the plasma membrane (Fig. 5). The K1250M and D1370N mutants impair the ATP hydrolysis at NBD2 without affecting the ATP binding, whereas the G1349D impairs the ATP binding. Therefore, these results indicate that the ATP-hydrolyzing conformational state of the second nucleotide-binding domain of CFTR is essential for its regulatory function toward VSOR Cl⁻ channels.

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Effect of EGF receptor pathway activation on VSOR chloride conductance in murine mammary gland C127 cells

The C127 cell line was originally derived from mammary glands of mouse. These cells have fibroblast-like morphology and are sensitive to the papillomaviruses (Law et al., 1981). The bovine papillomavirus (BPV)-mediated transfection was widely used to introduce foreign molecules into the cells, including CFTR. In the present study, we investigated the effect of BPV-mediated stable expression of CFTR protein on the properties of volume-sensitive outwardly rectifying (VSOR) Cl⁻ channels in C127 cells.

Up-regulation of VSOR Cl⁻ currents in C127 cells stably transfected with CFTR

In this study, we used CFTR-transfected C127 cells described in details by Marshall et al. (1994). No appreciable Cl⁻ current was detected in control non-transfected C127 cells (data not shown, n=3) in response to cAMP stimulation. However, in C127/CFTR cells, cAMP stimulation under isotonic conditions induced activation of Cl⁻ current with time-independent kinetics and linear I-V relationship (Figs. 8A, 8Ba and 8C), which are typical of CFTR Cl⁻ current. In Fig. 8, the same cell, when stimulated by hypotonic stress, developed a different kind of current with outward rectification and time-dependent inactivation at depolarizing potentials (Figs. 8A, 8Bb and 8C) which are typical of VSOR Cl⁻ current. Therefore, it appears that C127/CFTR cells express both functional CFTR and VSOR chloride channels. Figure 9A shows the time course of activation of VSOR Cl⁻ currents recorded from these cell lines after hypotonic stimulation. The activation kinetics was rather slow, and maximal steady-state

currents were at the level of 49.1 ± 7.2 pA pF^{-1} (n=11) for C127 cells. Time dependent inactivation at positive potentials (Fig. 9B) and outward rectification (Fig. 9D) are phenotypic characteristics of VSOR chloride currents. Similar Cl$^-$ currents were observed in C127/CFTR cells (Figs. 9A, B and D). The VSOR Cl$^-$ current in the CFTR-expressing cells differed from that of C127 cells in two respects. First, the activation kinetics was significantly faster; and, second, the peak Cl$^-$ current amplitude was app. 2.7 times larger in CFTR-transfected cells compared to that of control cells (Figs. 9C and D). This result is in contrast to the case of HEK293T cells (Fig. 7).

Faster kinetics and larger amplitudes of macroscopic VSOR Cl$^-$ current in C127/CFTR cells due to facilitated volume-dependent activation of single VSOR Cl$^-$ channels

Rapid activation of whole-cell current could be due to faster swelling rate (higher osmotic water permeability, P_f), altered single-channel parameters (single-channel amplitude and P_o) or altered volume sensitivity. In the cell-attached mode, stepwise closing single-channel events were observed at large positive potentials on osmotically pre-swollen cells exposed to high-K$^+$ hypotonic solution (Fig. 10). Time-dependent single-channel closing events at positive potentials (Fig. 10A), intermediate unitary conductance and outwardly rectifying current-voltage relationships (Fig. 10B) in both cell lines were similar to those described previously for swelling-activated, intermediate-conductance, outwardly rectifying chloride channels (Okada, 1997). The ensemble average of single-channel traces exhibited inactivation kinetics at positive potentials (data not shown), which was similar to the depolarization-induced inactivation of whole-cell VSOR Cl$^-$ currents. Since no single-channel closing events could be observed at moderately negative or positive potentials (±40 mV used for whole-cell measurements) in

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both control and transfected cells, we suggest that single-channel open probability was close to unity at these potentials. Therefore, we can exclude the possibility that CFTR expression modulates VSOR Cl⁻ channels by influencing it's P_o. On the other hand, the unitary conductance of VSOR single-channel in control cells (around 47 pS at +100 mV) was less than in CFTR-transfected cells (around 57 pS at +100 mV), as shown in Fig. 10. Although these values were significantly different ($p <0.001$), they could not explain 2.7 times larger whole-cell currents in C127/CFTR cells.

Cell swelling rate, and, hence, osmotic water permeability of the two cell lines did not differ significantly (Fig. 11A). In contrast, when the current density was plotted as a function of the relative cell volume (measured simultaneously during whole-cell patch-clamp experiments), I observed a strikingly large difference in volume sensitivity of whole-cell currents recorded from these two cell lines (Fig. 11B). Cl⁻ currents in C127/CFTR cells were significantly more sensitive to cell volume expansion than those in C127 cells.

C127/CFTR cells may have different resting intracellular pH (Gottlieb and Dosanjh, 1996; Luckie et al., 2001), which in turn may change the VSOR single-channel amplitude (Sabirov et al., 2000). However, in our experiments, the intracellular pH was essentially the same in the two cell lines and did not change significantly upon hypotonic stimulation (Fig. 12).

Taken together, our data strongly suggest that enhanced VSOR Cl⁻ channel activity in CFTR-expressing cells is only in part due to a larger single-channel conductance and mostly due to enhanced volume sensitivity of VSOR Cl⁻ channels.

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CFTR expression-induced up-regulation of VSOR Cl⁻ currents mimicked by transfection with ΔF508 mutant of CFTR and vector alone

As shown in Fig. 13, expression of ΔF508 mutant of CFTR led to the same level of up-regulation of VSOR Cl⁻ channel, as WT-CFTR. Surprisingly, empty vector-transfected cells (C127/BPV) also exhibited similar up-regulation of VSOR Cl⁻ currents (Fig. 13). First, I draw the conclusion that enhanced VSOR Cl⁻ channel activity observed in C127/CFTR cells reflects a change in the cellular physiological state induced by transfection rather than a specific effect of CFTR protein. Second, given strong down-regulating effect of CFTR expression observed in HEK293T cells (Fig. 3), and that observed in COS cells by Vennekens et al. (1999), I conclude that stable transfection of C127 cells using BPV genome-containing expression vector conferred CFTR-insensitivity on C127 cells.

In order to generate C127 cell lines stably expressing CFTR protein, Marshall et al. (1994) used mammalian expression vectors containing a full sequence of bovine papillomavirus genome (pBPV), which was expected to facilitate episomal maintenance of the plasmid in C127 cells. On the other hand, it is known that expression of some BPV proteins (like a small 44 kDa E5 protein) results in constitutive activation of platelet-derived growth factor (PDGF) and/or epidermal growth factor (EGF) receptors (Martin et al., 1989; Lai et al., 2000), which belong to the receptor tyrosine kinase family. Given that VSOR Cl⁻ channels are sensitive to tyrosine phosphorylation (Voets et al., 1998), I hypothesized that transfection of C127 cells with mammalian expression vectors containing BPV genome may result in expression of some viral proteins and consequent permanent activation of receptor tyrosine kinase signaling pathways.

Up-regulation of VSOR Cl⁻ currents by EGF but not PDGF in C127 cells

As shown in Fig. 14A (top panel, left), application of EGF (50 ng/ml) to C127 cells, after swelling-induced whole-cell Cl⁻ currents reached the steady-state level, led to further enhancement of VSOR Cl⁻ current. No effect was observed when C127/BPV cells (transfected with vector alone) were subjected to EGF (Fig. 14A, top panel, right). Bath application of PDGF (20 ng/ml) did not produce any effect on steady-state VSOR Cl⁻ currents both in C127 cells and in C127/BPV cells (Fig. 14A, bottom panel). As summarized in Fig. 14B, only EGF significantly up-regulated VSOR Cl⁻ currents in C127 cells, but not in C127/BPV cells.

An inhibitor of EGF receptor tyrosine kinase-mediated phosphorylation, tyrphostin B46, inhibited whole-cell VSOR Cl⁻ currents in C127 cells (Fig. 15A), whereas a specific inhibitor of PDGF receptor phosphorylation, tyrphostin AG1296, did not affect the currents (data not shown).

If stable transfection with BPV-containing vector leads to constitutive activation of receptor tyrosine kinases, then C127/BPV cells are expected to be more sensitive to a specific inhibitor for the receptor kinase constitutively activated in these cells. Indeed, as shown in Fig. 15B, the dose-response curves of tyrphostin B46 yielded the IC_{50} values of 31 μM for whole-cell VSOR Cl⁻ currents in control C127 cells and 12 μM for those in C127/BPV cells. Note that Hill coefficient (h) was about the same for the two cell lines, indicating that the stoichiometry of receptor-inhibitor interaction was not affected by stable transfection.

Tyrphostin B46-induced inhibition was not due to a direct blocking of the channel, since the single-channel amplitude was not altered by tyrphostin B46 (Fig. 15C).

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Regulatory volume decrease is sensitive to an EGF receptor inhibitor.

Since the main physiological function of the VSOR Cl⁻ channel is to provide a conductive pathway for anion extrusion during volume regulation, the regulatory volume decrease (RVD) is expected to be sensitive to the blockers of VSOR Cl⁻ currents. Therefore, we tested if the RVD in C127 cells is sensitive to the inhibitors of receptor tyrosine kinases. As shown in Fig. 16, the volume recovery after cell swelling in response to a hypotonic challenge was considerably impaired in C127/BPV cells by application of tyrphostin B46, but not tyrphostin AG1296. Therefore, the RVD process displayed the same pharmacological pattern as the VSOR Cl⁻ current with respect to receptor tyrosine phoshorylation.

PCR analysis.

To examine expression of the EGFR gene, RT-PCR was performed on RNA isolated from C127 cells. As shown in Fig. 17, the PCR product of the expected length, i.e. 320 base pair, could be amplified by mouse EGFR-specific primers from reverse-transcribed RNA (lane *EGFR primer*). However, no PCR product was amplified when the cDNA (data not shown) or the reverse transcriptase (lane *No RT*) were omitted from the reaction. G3PDH, a house-keeping protein with abundant expression, was used as a positive control (lane *G3PDH*). The PCR product revealed >97% identity of the nucleotide sequence with the corresponding region of mouse EGFR (Fig. 17). Since no PCR product could be amplified using 1 round 25 cycles (data not shown), it might be possible, that the EGF receptor is expressed in C127 cells at a low level.

DISCUSSION

CFTR-mediated channel regulation by the protein-protein interaction

The first purpose of my study was to investigate the relationship between cystic fibrosis transmembrane conductance regulator (CFTR) and volume-sensitive outwardly rectifying (VSOR) chloride channels. The VSOR Cl$^-$ channel activity was found to be greatly down-regulated by CFTR protein expression in a HEK293T cell line. The regulatory effect was completely abolished by a common CF mutation, ΔF508, suggesting importance of plasma membrane expression of this protein. This result is in good agreement with the data obtained in CPAE and COS cell lines by Vennekens et al. (1999) and further supports the idea that CFTR-VSOR interaction is not cell type-specific. Since the regulation occurred without stimulation by PKA, I suggest that it is not related to the function of CFTR as a chloride channel, but rather reflects its activity as a channel regulator.

Outwardly rectifying chloride channels, ORCC, epithelial sodium channels, EnaC and renal epithelial potassium channels (ROMK1, ROMK2 and Kir6.1) are channels, which can be regulated by CFTR. Does CFTR regulate the VSOR channel by a similar mechanism?

ORCC (outwardly rectifying chloride channel) is highly expressed in airway epithelia and resembles the VSOR chloride channel with respect to its intermediate single-channel conductance and outward rectification. ORCC is also controlled by CFTR expression, although, in contrast to the VSOR Cl$^-$ channel, it is augmented (not suppressed) by CFTR. The CFTR-dependent PKA-mediated

activation of ORCC was preserved in lipid bilayer reconstitution experiments (Jovov et al., 1995a, b), hinting on the direct protein-protein interaction. However, the current hypothesis involves CFTR-mediated ATP release followed by extracellular ATP-binding either to the channel itself or to a purinergic receptor in up-regulation of ORCC by CFTR (Schwiebert et al., 1998, 1999). Expression of C- and N-terminal truncated forms of CFTR into a human CF airway epithelial cell line, IB3-1, or in *Xenopus* oocytes revealed that the NBD1 domain and the R domain are essential for its ability to regulate ORCC, while a chloride conductance was abolished by dual-arginine mutation R334W/R347P in pore-forming TM6. The natural splice-variant with stop-codon at D835X retained only the first half of molecule (first six TMs along with NBD1 and R domains) was also able to generate functional chloride channels and regulate ORCC, indicating that neither the NBD2 domain nor the C-terminus (which is essential for the PDZ-domain interaction of CFTR) is involved in ORCC regulation by CFTR. The G551D mutant of CFTR, which produces severe lung disease in 3% of all CF patients, is normally processed to the plasma membrane, but does not generate a functional chloride conductance due to impaired ATP-binding at NBD1 (Yang et al., 1993). This point mutation completely abolished the ORCC regulation by CFTR both in airway epithelial cells (Fulmer et al., 1995) and in planar lipid bilayers (Jovov et al., 1995b).

In cystic fibrosis, amiloride-sensitive sodium absorption in airway epithelia was greatly increased owing to the release from CFTR-mediated down-regulation of ENaC (Boucher et al., 1986). The ENaC channel protein alone, when expressed in MDCK cells exhibited a Na^+ conductance, and it was further up-regulated by cAMP stimulation. However, when coexpressed with CFTR, the Na^+ currents became smaller and inhibited (not activated) by cAMP (Stutts et al., 1995). Therefore, CFTR inverted PKA-dependent regulation of ENaC, as it was seen also at the single-channel level (Stutts et al., 1997). Coexpression of truncated forms of

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CFTR molecule showed that the C-terminal half is not essential for ENaC inhibition, whereas functional NBD1 was necessary (Kunzelmann et al., 1997; Schreiber et al., 1999; Kunzelmann et al., 2000). As was the case of ORCC, the G551D CF mutation in NBD1 abolished the CFTR-mediated regulation of ENaC both in oocytes (Kunzelmann et al., 1997) and in planar lipid membranes (Ismailov et al., 1996). Immunopurified CFTR was able to inhibit immunopurified amiloride-sensitive Na^+ channel and recombinant ENaC channels in lipid bilayers (Ismailov et al., 1996; Berdiev et al., 2000), suggesting a direct protein-protein interaction between the two molecules. The protein-protein interaction detected by a yeast two-hybrid analysis was found to be sensitive to G551D mutation (Kunzelmann et al., 1997). However, the fact that CFTR-dependent up-regulation (not inhibition) of sodium reabsorption was observed in sweat ducts (Reddy et al., 1999) implies a more complex relationship between CFTR and ENaC.

Coexpression of the renal epithelial potassium channels, ROMK1, ROMK2 and Kir6.1, was found to increase sensitivity to a sulfonylurea, glibenclamide (Ruknudin et al., 1998; McNicholas et al., 1996, 1997; Ishida-Takahashi et al., 1998). For ROMK2, CFTR truncation after the first NBD domain had no significant effect on the CFTR-ROMK2 interaction (McNicholas et al., 1997), though the R-domain was necessary for the PKA-dependent attenuation of ROMK2-CFTR complex (Cahill et al., 2000). In the meantime, it was found that, when CFTR was further shortened to exclude the NBD1 portion, the glibenclamide sensitivity was lost completely (Cahill et al., 2000), implying that the first nucleotide-binding fold is crucial for ROMK2 regulation by CFTR. Furthermore, a severe CF mutation G551D abolished the CFTR-dependent component of glibenclamide-induced inhibition, while a milder form of CF mutation in the same NBD1, A455E, did only partially (McNicholas et al., 1997). Note that the A455E mutation exhibited a similar mild effect on the CFTR-ORCC interaction (Fulmer et al., 1995).

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ORCC, ENaC and ROMK2 channels can thus be collectively grouped as NBD1-CFTR-dependent channels. Even though features of CFTR-mediated regulation are different in all three cases (activation for ORCC, inhibition for ENaC, and glibenclamide-sensitivity for ROMK2), the modulation itself is due to a direct or indirect interaction with the NBD1 domain and is sensitive to the G551D mutation. Although any point mutations in the second half of CFTR were not tested in these studies, the truncation of this part had no effect on the interaction between CFTR and these three types of channel (Cahill et al., 2000, Kunzelmann et al., 1997). The results obtained in the present study showed that the VSOR chloride channel differs markedly from these three channels in one important point: the CFTR-mediated regulation of VSOR Cl⁻ channel was insensitive to a severe G551D CF mutation in NBD1. On the contrary, the G1349D mutation, which is also associated with a severe CF phenotype with pancreatic insufficiency, effectively impaired the CFTR-VSOR interaction. Moreover, other mutations in the putative ATP-binding pocket of NBD2, K1250M and D1370N, also abolished the CFTR-VSOR interaction. Thus, there is a possibility that conformational state of the ATP-binding pocket itself is essential for down-regulation of VSOR Cl⁻ channel by CFTR protein. It is tempting to speculate that abnormal electrolyte transport in some CF mutations in the NBD2 domain might be related to an aberrant volume-sensitive chloride channel activity and cell volume regulation important for secreting epithelia.

PDZ domains, initially identified in postsynaptic density-95 (PSD-95), disc large and ZO-1 proteins, are found in a variety of multifunctional proteins and responsible for protein-protein interactions in different structures such as PSD in neurons (Kornau et al., 1995; Brenman et al., 1996) and junctional complexes in epithelia (Ebnet et al., 2000). PDZ domain-mediated interactions were shown to facilitate clustering of ion channels within microdomains and the assembly of

signaling complexes at the plasma membrane. CFTR was shown to bind with high affinity to EBP50 (ERM-binding phosphoprotein 50) (Short et al., 1998; Moyer et al., 1999). This protein is expressed in many epithelial cells and localized at apical surface. Human EBP50 and its rabbit homolog, NHE-RF (NHE regulatory factor) can bind to the isomer of NHE3 Na^+/H^+ exchangers to confer the cAMP-dependent inhibition. Three amino acids, TRL (tyrosine-arginine-lysine), located at the C-terminus of CFTR represent a PDZ binding consensus motif, and truncation mutants ΔTRL and ΔDTRL were shown to lack the ability of binding to EBP50 (Moyer et al., 1999) and regulate the NHE activity (Ahn et al., 2001). A plausible assumption would be that VSOR regulation by CFTR is also mediated via PDZ domain binding. In our experiments, ΔTRL-CFTR mutant was able to suppress the VSOR Cl^- current to the same extent as the wild type. This result strongly suggests that the C-terminal PDZ-binding domain of CFTR, which is responsible for EBP50/NHE-RF-mediated regulation of sodium-proton exchange, is not involved in VSOR chloride channel regulation. A similar result was recently obtained also for Ca^{2+}-activated Cl^- channels (Wei et al., 2001) suggesting that CFTR uses different parts of its molecule for control of the ion channels and transporters.

Since neither NBD1 nor PDZ-binding domain of CFTR is involved in VSOR channel regulation, we may suppose that an intact structure of NBD2 domain either provides an appropriate conformation for its direct binding to VSOR channel protein or for an indirect regulation, for example via Syntaxin A-mediated binding to it's N-terminus.

Tyrosine kinase-mediated regulation of volume-sensitive Cl^- channel

The C127 cell line is derived from mouse mammary gland tumour and has long been used a recipient cell line for papillomavirus-induced transformation (Petti et al., 1991; Lai et al., 2000, Levenson et al., 1989). Bovine papillomavirus (BPV)

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genome containing plasmids were extensively used to introduce foreign molecules, including CFTR. Based on our experimental data obtained with HEK293T cells transfected with CFTR, we would expect a similar inhibitory effect of CFTR expression in C127/CFTR cells. However, the result was opposite: CFTR-expressing cells had app. 3 times higher VSOR Cl⁻ channel activity compared to the control cells. This enhanced channel activity resulted mainly from increased volume sensitivity of VSOR Cl⁻ channel and slightly increased single-channel amplitude. A common CF mutation, ΔF508, was found to be similarly effective in up-regulating the activity of VSOR chloride channel in these cells. Moreover, the cells transfected with BPV-vector alone (C127/Mock cells) exhibited a marked VSOR Cl⁻ channel activity which was comparable to those in C127/CFTR and C127/ΔF508 cells but significantly greater than that in parental C127 cells. Based on these observations, and given a prominent inhibitory effect of CFTR expression on the VSOR Cl⁻ current observed in three different cell lines (HEK293T cells in the present experiments, and CPAE and COS cells by Vennekens et al. 1999), I conclude that the VSOR chloride channel lost their sensitivity to CFTR in BPV-transfected C127 cells. When the expression vector containing full bovine papillomavirus genome was introduced into C127 cells during the transfection procedure (Marshall et al., 1994), marked morphological changes (including loss of apparent contact inhibition) were induced. Remarkably, this feature was common for the cells, stably transfected either by BPV alone or with WT-CFTR and ΔF508-CFTR mutant, but not for control C127 cells, indicating that the transfection procedure greatly altered the cellular physiological status of the transfectants. As I noted before, this cell line has long been used as a test for BPV-dependent cellular transformation, and the phenotype of transfected cells closely resembles the one described previously for BPV-transformed cells (DiMaio et al., 2000). Therefore I suppose that the viral proteins presumably

introduced into transfected cells were responsible for the change in the VSOR Cl⁻ channel activity in C127 cells.

What was the precise mechanism by which BPV-transfected cells could overcome the down-regulating effect of CFTR molecule on VSOR chloride channels? Short peptides, encoded in BPV, induce C127 cell transformation (DiMaio et al., 1986; Settleman et al., 1989) via growth factor-independent constitutive activation of PDGF receptors (for review, see DiMaio et al., 2000). Therefore, I checked the possibility that BPV-induced PDGF receptor activation is involved in augmentation of swelling-induced chloride current. However, neither a PDGF peptide itself nor the specific inhibitor of PDGF-receptor phosphorylation, tyrphostin AG1296, was able to affect whole-cell VSOR Cl⁻ currents, implying that PDGF receptor activation was not involved in volume-sensitive chloride channel regulation.

Papillomavirus may also enhance and/or constitutively activate the EGF receptor (Martin et al., 1989). Therefore, we tested the hypothesis of EGF-receptor tyrosine kinase involvement in a mediator of an altered VSOR Cl⁻ channel regulation in transfected C127 cells. Indeed, I found that an EGF peptide could significantly activate the VSOR Cl⁻ currents in control C127 cells, but not C127/BPV cells where VSOR Cl⁻ channels are supposedly already up-regulated by BPV proteins. Furthermore, tyrphostin B46, a relatively specific inhibitor of EGF-receptor tyrosine kinase, had a profound effect on VSOR Cl⁻ currents in both C127 and C127/BPV cells. The latter cells were more sensitive to tyrphostin B46 possibly due to the higher activation status of EGF-receptor in BPV-mediated transfected cells. Thus, our data suggest that when C127 cells were transfected with an expression vector containing the full genome of BPV, VSOR chloride channels expressed in these cells were freed from down-regulation by CFTR protein owing

to the activated status of tyrosine kinase signaling cascade, most likely involving activation of EGF-receptor tyrosine kinase. Note that the Kv1.3 potassium channel, when coexpressed with the recombinant EGF-receptor, acquired EGF-dependent down-regulation (Bowlby et al., 1997). Similar inhibitory effects of agonists for EGF-, FGF- and PDGF-receptors were observed in PC12 cells (Hilborn et al., 1998).

The EGF receptor (EGFR) is a cell surface receptor with intrinsic protein tyrosine kinase activity. The unstimulated EGFR is a monomer, which can undergo dimerization upon activation by extracellular ligand. Dimerization leads to the autophosphorylation of its cytoplasmic domain at six tyrosine phosphorylation sites. Several adapter proteins are recruited at these sites. Figure 18 summarizes possible EGFR signaling pathways.

PLCγ binds directly to the EGFR via its SH2 domain (Bogdan and Klambt, 2001). When activated, PLC is able to cleave PI(4,5)P$_2$ to generate inositol triphosphate (IP$_3$) and diacylglycerol (DAG), which in turn activates protein kinase C (PKC). To check, whether the VSOR Cl$^-$ channel is regulated by EGF receptor via PLCγ-pathway, I applied a PLC inhibitor, U73122 (50 μM). However, the VSOR Cl$^-$ current recorded at steady-state level in the whole-cell mode was never affected by this drug (data not shown).

Another adapter protein which is recruited and phosporylated by EGFR, is the p85 subunit of phosphatidylinositol-3-kinase (PI3K) (Moghal and Sternberg, 1999). PI3K was shown to be transiently activated upon cell swelling (Tilly et al., 1996). To investigate the role of this kinase in VSOR Cl$^-$ current activation, I added wortmannin (5 μM), an inhibitor of PI3K (Ui et al., 1995), to the hypotonic solution. At the steady state level of VSOR Cl$^-$ current, again, no effect of this drug was found (data not shown).

The next downstream signaling cascade of the EGFR is the MAP kinase pathway. It is initiated by phoshorylation of an adapter protein GRB2 and leads to subsequent activation of the small G-protein Ras, which, in turn, stimulates the MAP kinase pathway (Bogdan and Klambt, 2001). When PD98059, a selective and cell-permeable inhibitor of MAP kinase kinase (MEK) that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates (Dudley et al., 1995), was added to the hypotonic solution after VSOR Cl$^-$ currents reached the steady-state level, no effect was observed (data not shown).

These preliminary data indicates that it might be possible that EGFR-mediated regulation of VSOR Cl$^-$ channel in C127 cells involves some downstream signaling events other than those distal to PLCγ, PI3K and MEK.

Effects of EGF and a specific inhibitor of EGF receptor autophosphorylation suggest that the enhanced VSOR Cl$^-$ current activity observed in C127/CFTR cells is (at least in part) due to constitutive activation of the EGF receptor tyrosine phosphorylation signaling pathway. Since a number of receptors and their signaling molecules are known to be associated with caveolae (see Okada, 1999), I then performed the morphological study of the cells by transmission electron microscopy. Figure 19 shows electron micrographs obtained from preparations of control C127 cells and stable transfectants of CFTR (C127/CFTR), ΔF508 (C127/ΔF508) and vector alone (C127/BPV). A striking feature of all transfected cells, but not the control cells, is the presence of large number of caveolae on their surface plasma membrane. C127 cells had a very small amount of caveolae, and arrows in the graph pointed at the only places containing caveolae.

An EGF-dependent MAP kinase activation requires the recruitment of Raf-1, a kind of MEKK, to the plasma membrane. It has been shown that caveolae are the

membrane site where Raf-1 is recruited (Mineo et al., 1996). Since the expression of caveolin-1, a main scaffolding protein of caveolae, in caveolin-deficient cell lines leads to a strong up-regulation of the endogenous VSOR Cl^- channels (Trouet et al., 1999), it might be possible that EGFR up-regulates VSOR Cl^- currents somehow by the recruitment of Raf-1 to caveolae.

VSOR Cl^- currents in C127/CFTR cells were indistinguishable from those in BPV-expressing cells, indicating that BPV expression might abolish CFTR-induced VSOR down-regulation. This effect might be explained by the next possibilities:

a) VSOR might be preferentially targeted to caveolae, and CFTR is not. Then, these two proteins may be spatially separated by a possible recruitment of more VSOR proteins more preferentially to caveolae;

b) if CFTR-VSOR interaction is due to an intermediate accessory protein, then a constitutive up-regulation of the growth factor receptor signaling pathway could phosphorylate an accessory protein, thereby leading to it's conformational state required for the down-regulation of the VSOR channel by CFTR.

In conclusion, the VSOR chloride channel is down-regulated due to NBD2-mediated control by the CFTR protein in epithelial cells. At the same time, the VSOR chloride channel is controlled by tyrosine phosphorylation and may serve as a target protein for the EGF-receptor tyrosine kinase signaling cascade. The CFTR-VSOR protein interaction would presumably be impaired either by tyrosine phosphorylation of the VSOR Cl^- channel protein (or its accessory protein) and/or by its interaction with scaffolding proteins, such as caveolins.

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A

Mock

B

WT-CFTR
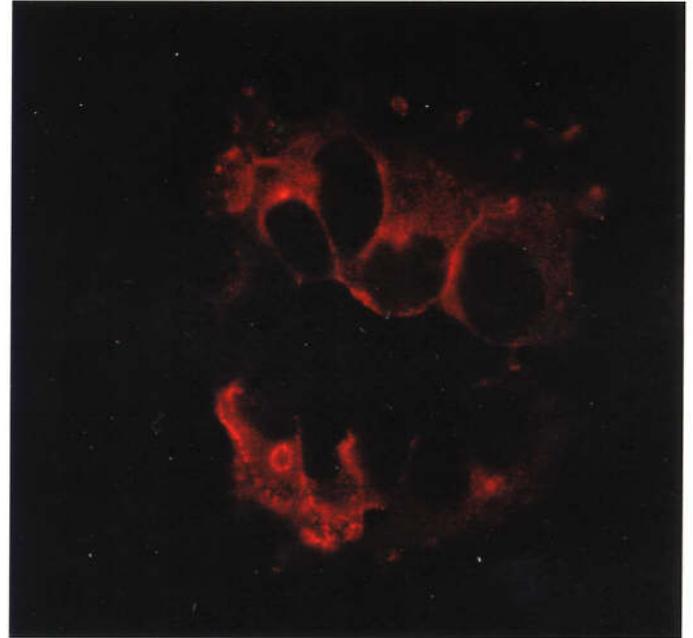
Fig.1. Confocal scanning microscope images of HEK293T cells visualized using anti-CFTR antibody raised against the intracellular R-domain of CFTR.

A, Fluorescence photographs of Mock-transfected cells. *B*, Fluorescence photographs of WT-CFTR-transfected cells. CFTR-immunostaining shows equable expression of the protein on the plasma membrane.
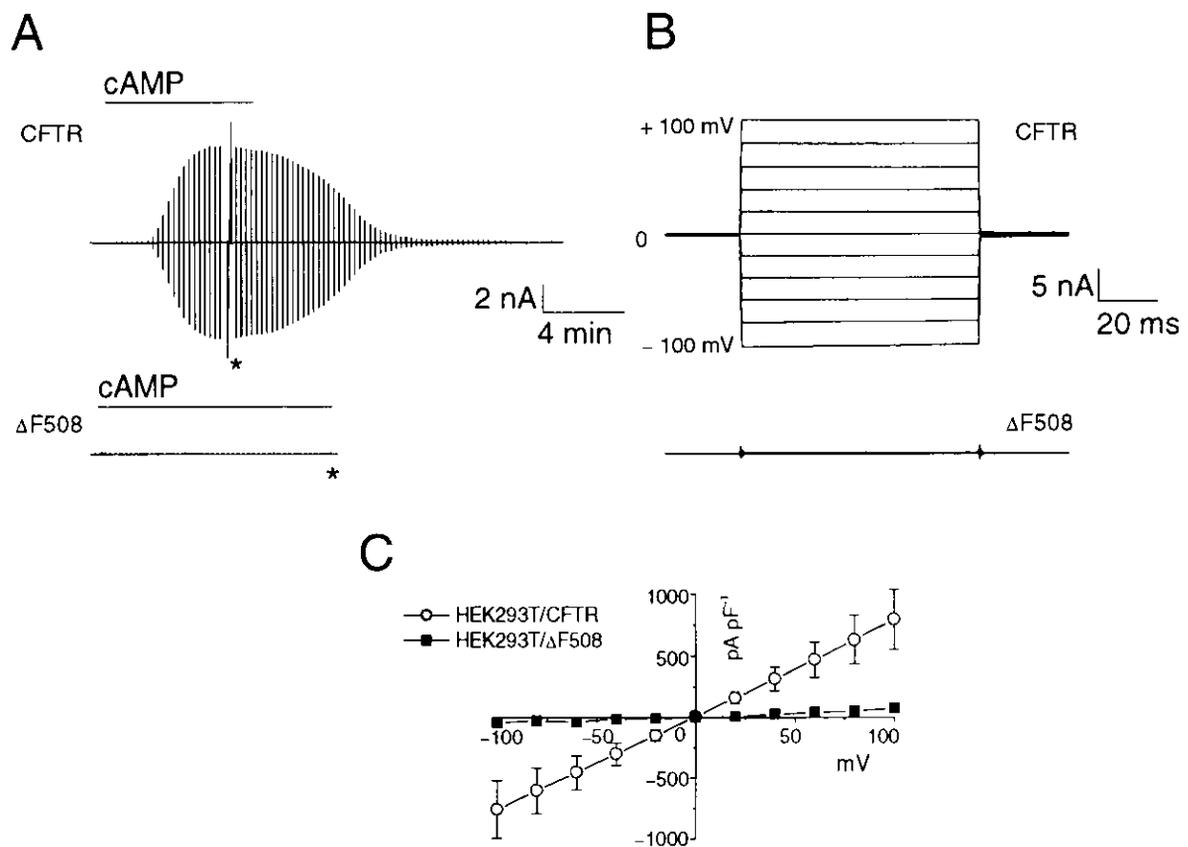
Fig. 2. Expression of cAMP-activated CFTR Cl⁻ currents in HEK293T cells transfected with WT-CFTR, but not with ΔF508. *A,* representative records before and after application of forskolin/dbcAMP/IBMX cocktail, during application of alternating pulses from 0 to ±40 mV. *Top,* HEK293T/CFTR; *bottom,* HEK293T/ΔF508 cells. *B,* representative records in response to step pulses (asterisks) from −100 to +100 mV. *Top,* HEK293T/CFTR; *bottom,* HEK293T/ΔF508 cells. *C,* I-V relationships for the mean cAMP-activated current densities.
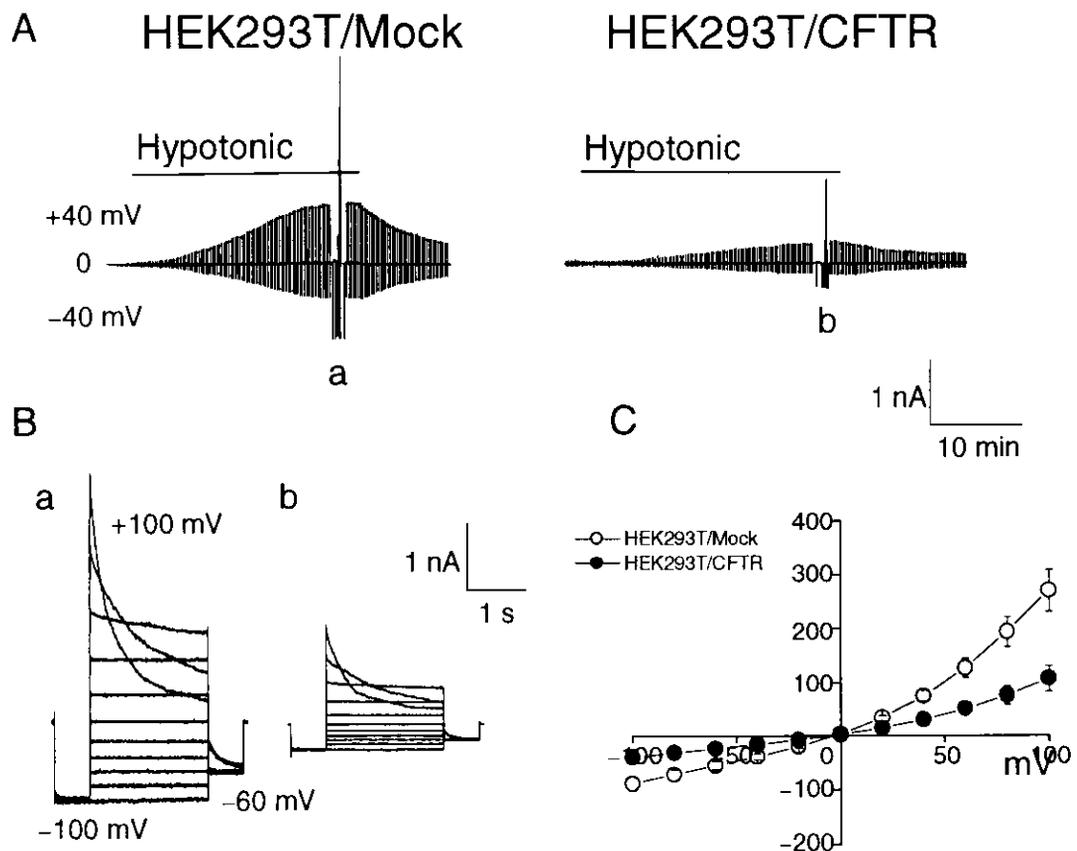
Fig. 3. Expression of CFTR inhibited VSOR Cl⁻ current activation in HEK293T cells. *A*, representative record before and after hypotonic challenge during application of alternating pulses from 0 to ±40 mV or of step pulses (*a, b*) from −100 to +100 mV in 20-mV increments in HEK293T/Mock and HEK293T/CFTR cells. *B*, expanded traces of current responses (*a, b* in *A*) to step pulses from −100 to +100 mV under hypotonic stress. *C*, I-V relationships for the mean VSOR Cl⁻ current densities. Each symbol represents the mean value (bar: s.e.m.) of 7 observations.
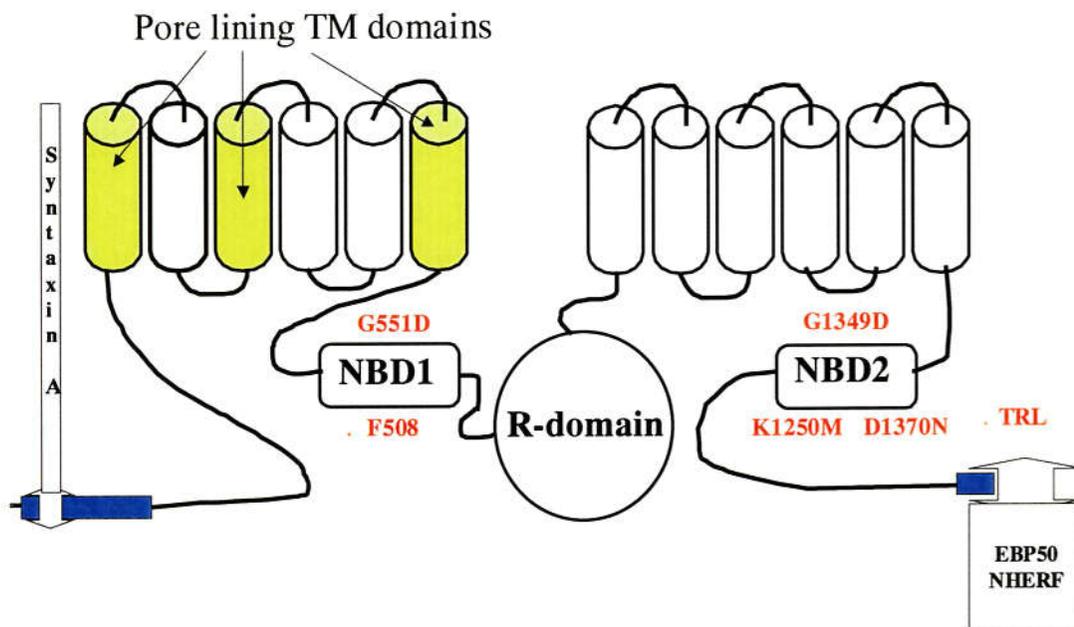
Fig. 4. Membrane topology of CFTR protein and the mutants examined in this study. Putative pore-lining trans-membrane domains are in green. The N-terminal portion is responsible for binding to Syntaxin proteins, and the C-terminal amino acids (TRL) are involved in binding to PDZ-domain-containing proteins. Positions of mutations tested are indicated in red.
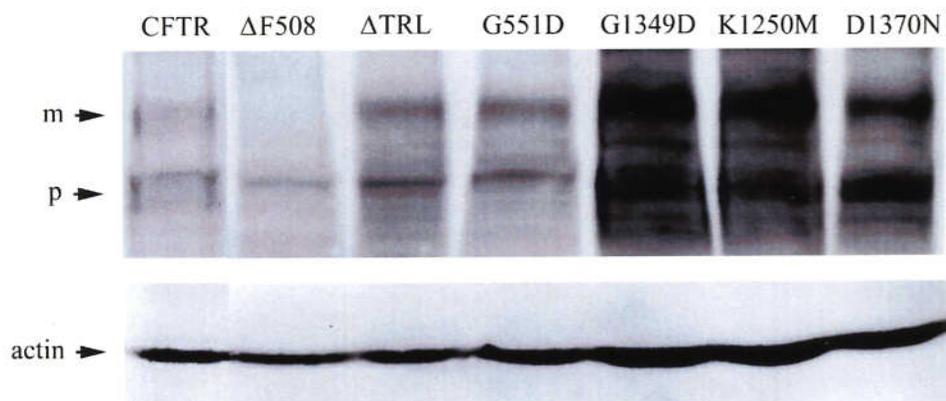
Fig. 5. Expression of CFTR and CFTR mutants other than ΔF508 in the plasma membrane of HEK293T cells evidenced by Western blotting.
m and p represent the mature (glycosylated) plasma-membrane CFTR and premature (unglycosylated) CFTR proteins, respectively. Actin bands are also presented as the internal control.

ΔF508
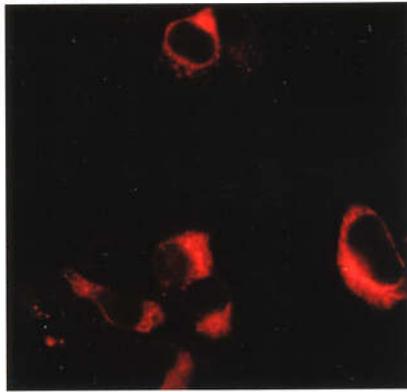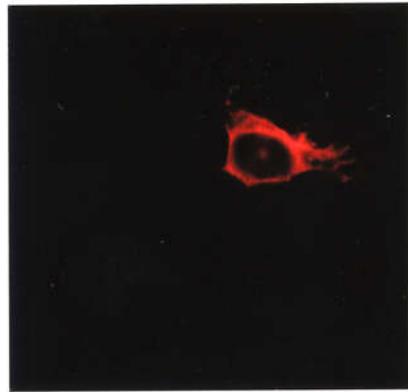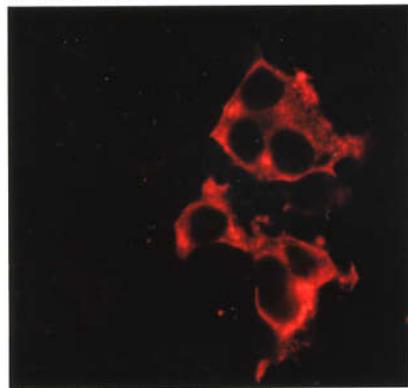
G551D

D1370N

ΔTRL
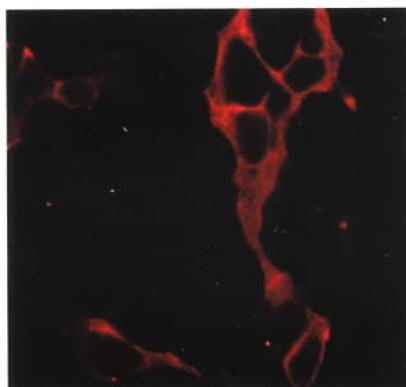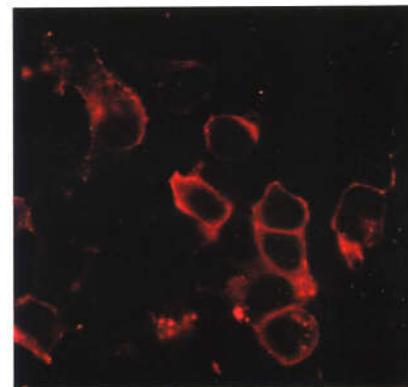
G1349D

K1250M

Fig. 6. Laser scanning microscopy images of HEK293T cells transfected with ΔF508, G551D, G1349D, ΔTRL, K1250M and D1370N mutants.
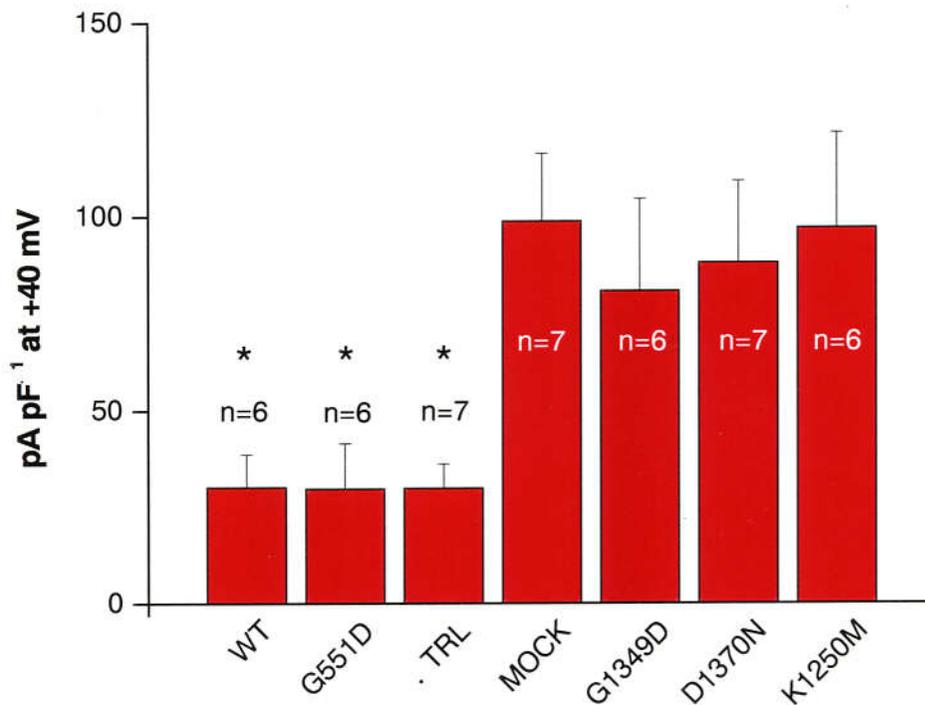
Fig. 7. Effects of expression of WT-CFTR, G551D, . TRL, G1349D, K1250M and D1370N mutants on VSOR Cl current densities in HEK293T cells. Graph shows VSOR Cl current densities at +40 mV after reaching a steady-state level. VSOR Cl current densities in HEK293T cells transfected with WT-CFTR or its mutant were compared with VSOR Cl current densities in empty vector-expressing (Mock) HEK293T cells. Asterisks, $p < 0.05$.
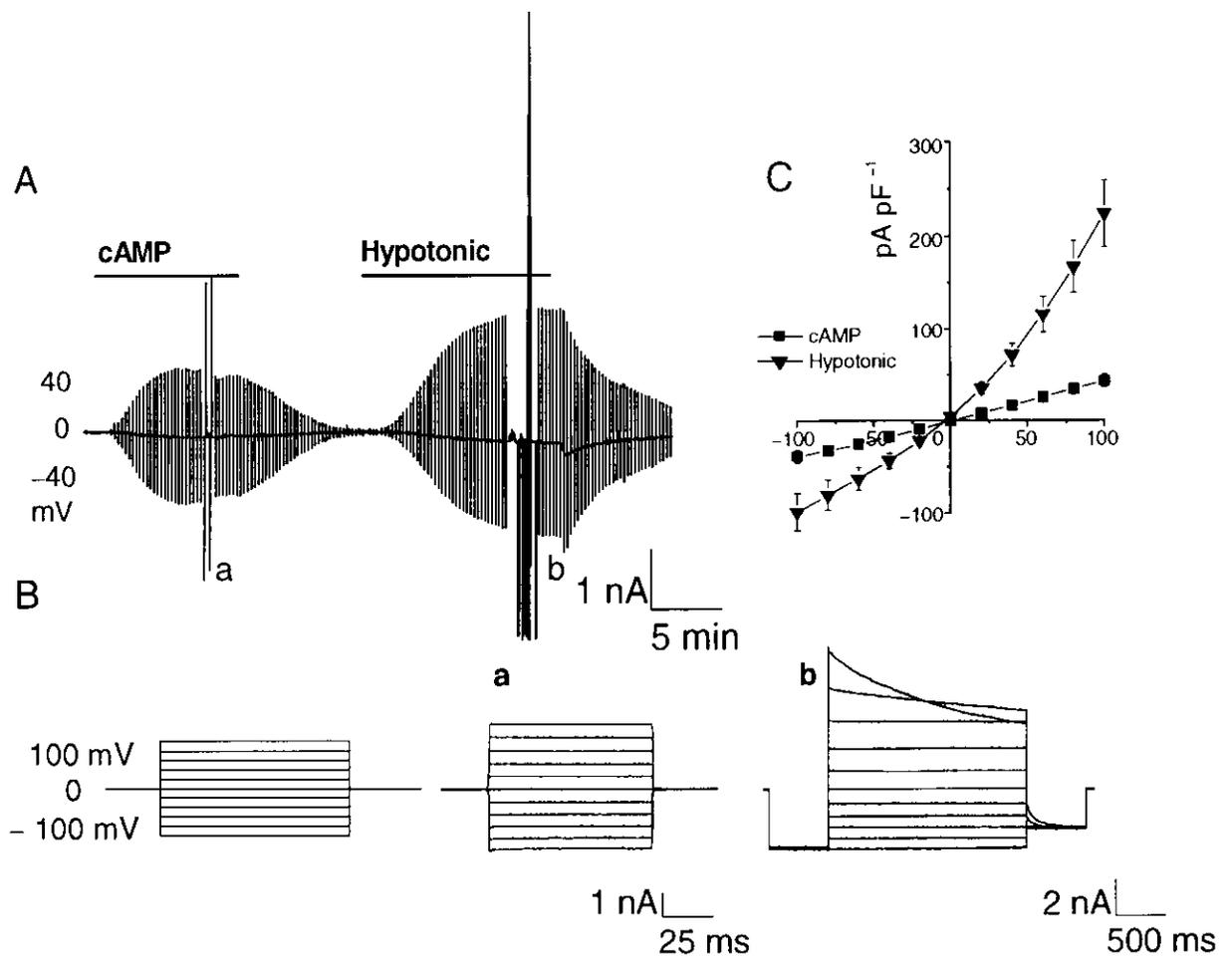
Fig. 8. **C127/CFTR cells showed functional expression of both cAMP-activated (CFTR) and volume-sensitive outwardly rectifying (VSOR) Cl⁻ currents.** *A*, representative record before and after a hypotonic challenge, or addition of a forskolin/dbcAMP/IBMX cocktail, during application of alternating pulses from 0 to ±40 mV or of step pulses from −100 to +100 mV (*a* and *b*). *B*, expanded traces of current responses (*a* and *b* in *A*) to step pulses from −100 to +100 mV upon stimulation with a forskolin/dbcAMP/IBMX cocktail (*a*) or a hypotonic challenge (*b*). *C*, I-V relationships for the mean VSOR Cl⁻ current densities ($n=11$, filled triangles) and CFTR Cl⁻ current densities ($n=13$, filled squares).
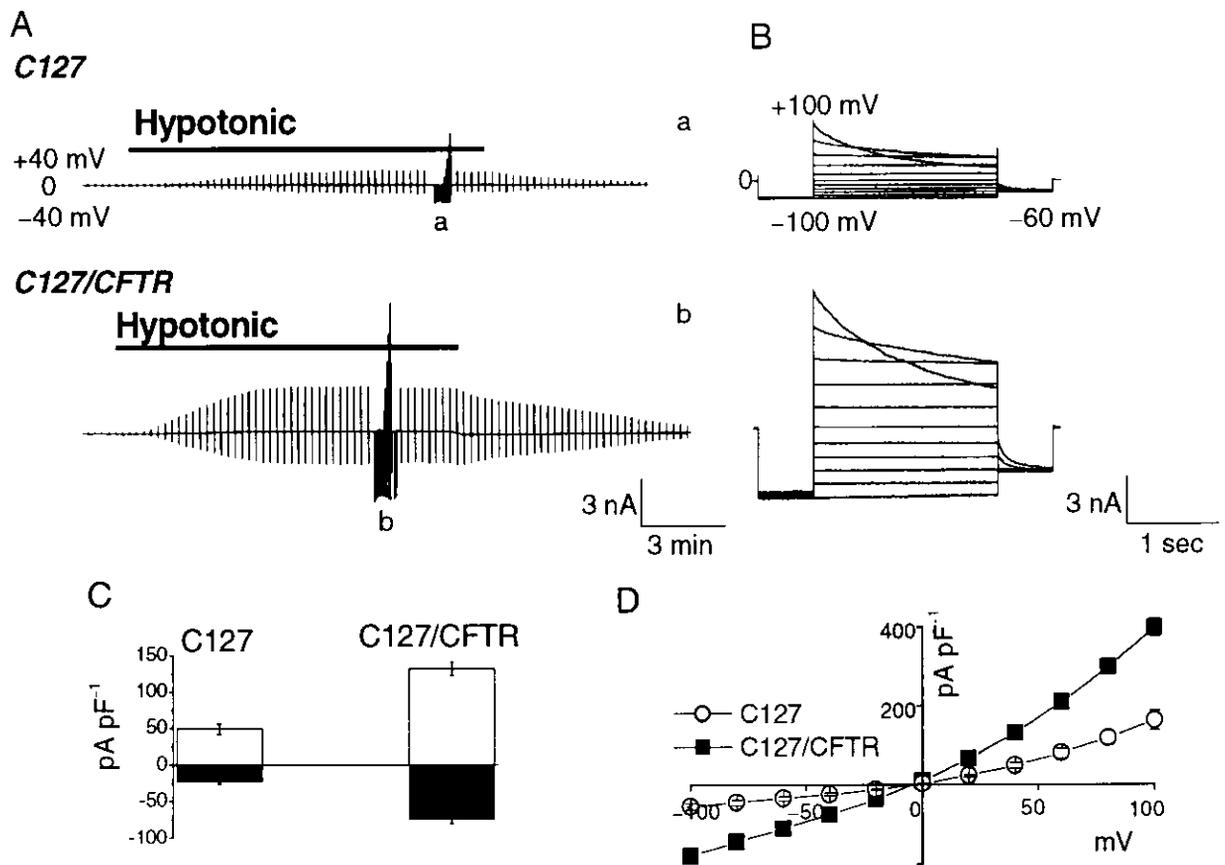
Fig. 9. VSOR Cl⁻ currents were up-regulated by BPV-mediated CFTR expression in C127 cells. *A*, Representative record before and after a hypotonic challenge during application of alternating pulses from 0 to ±40 mV or of step pulses from −100 to +100 mV with 20-mV increments (at a and b) in C127 (upper panel) and C127/CFTR cells (bottom panel). *B*, expanded traces of current responses to step pulses (a and b in A). *C*, Mean VSOR Cl⁻ current densities (bar: s.e.m.) recorded at ± 40 mV in C127 (open columns, $n=12$, -22.7 ± 12.1 pA pF⁻¹ at −40 mV) and C127/CFTR cells (filled columns, $n=18$, -74.5 ± 5.6 pA pF⁻¹ at −40 mV). *D*, I-V relationships for mean VSOR Cl⁻ current densities in C127 ($n=12$, open circles) and C127/CFTR cells ($n=18$, filled squares).
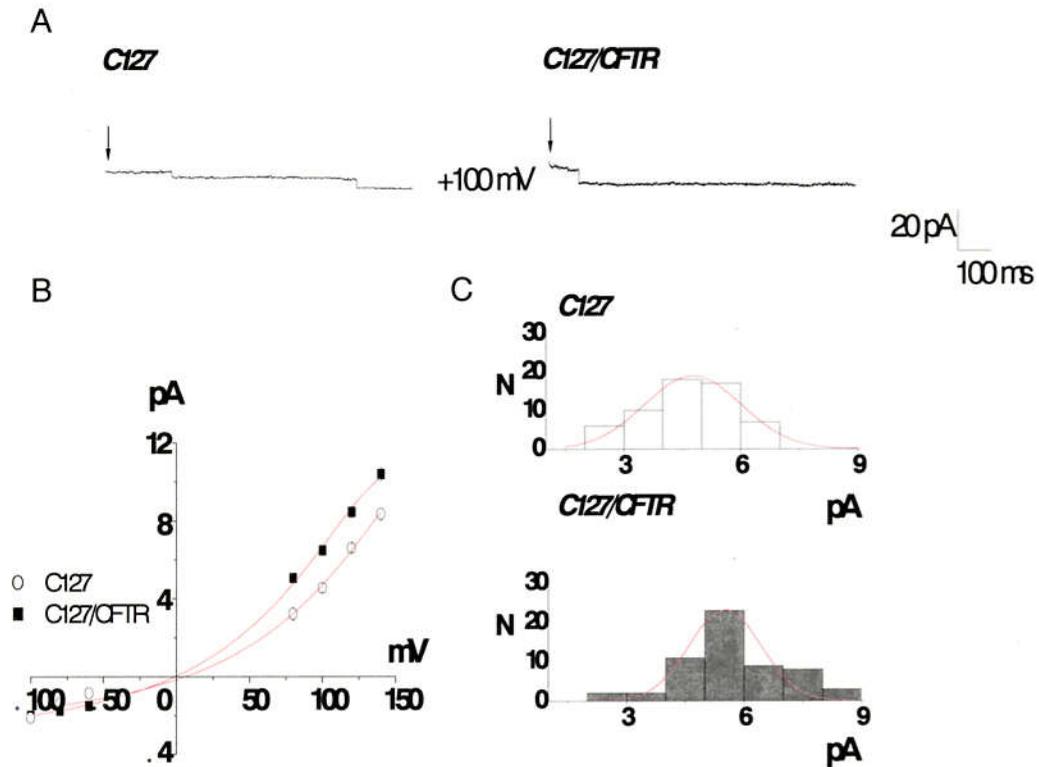
Fig. 10. VSOR single-channel conductance was up-regulated by BPV-mediated CFTR expression in C127 cells. *A,* VSOR single-channel recordings upon applying a step pulse (at arrows) from a holding potential of –140 mV to +100 mV (. V_p) in C127 (left panel) and C127/CFTR cells (right panel) in the cell-attached mode. *B,* I-V relationships of the VSOR single-channel currents in C127 (open circles, *n*=3-20) and C127/CFTR cells (filled squares, *n*=3-16). *C,* Amplitude histogram plots for VSOR single-channel currents recorded at +100 mV in C127 (upper panel, *n*=58, . = 46.7. 1.4 pS) and C127/CFTR cells (bottom panel, *n*=58, . = 57.0 . 1.7 pS).
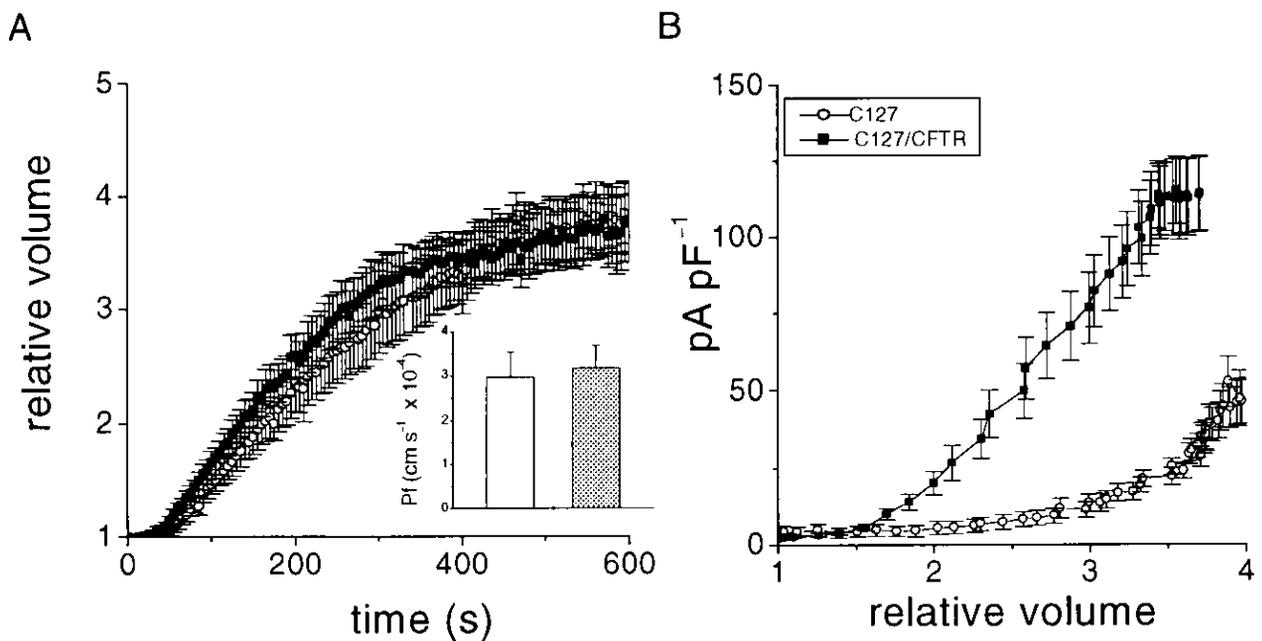
Fig. 11. Volume expansion sensitivity of VSOR Cl⁻ channel, but not osmotic water permeability, was enhanced in C127/CFTR cells compared to that in C127 cells. A, Osmotic swelling rate in C127 (open circles) and C127/CFTR (filled squares) are not statistically different between each other. Each symbol represents the mean value of 8 cells (with s.e.m.: bar). Inset: Osmotic water permeability is also not significantly different between C127 (white column) and C127/CFTR cells (grey column). B, Whole-cell VSOR Cl⁻ current densities are plotted as a function of respective relative volume changes in C127 (open circles) and C127/CFTR (filled squares) cells under the whole-cell configuration.
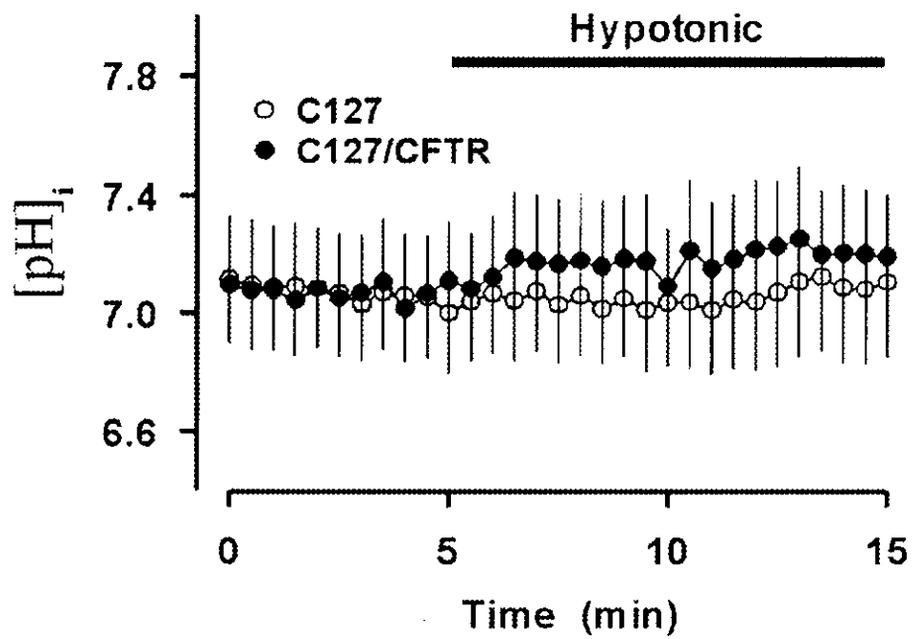
Fig. 12. Intracellular pH was not altered by BPV-mediated CFTR expression in C127 cells. Intracellular pH was measured using BCECF dye fluorescent method before and after hypotonic stress (83% osmolality). Open circles, C127 cells ($n=8$). Filled circles, C127/CFTR cells ($n=9$).
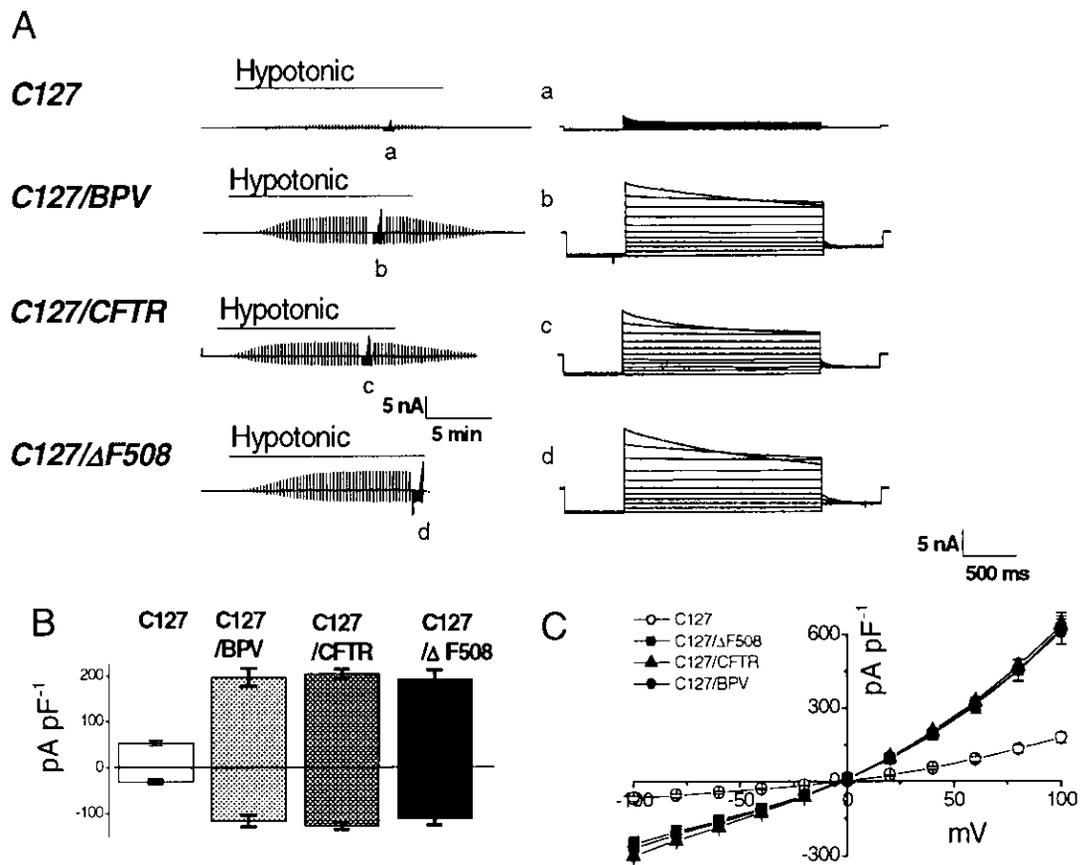
Fig. 13. BPV-mediated expression of ΔF508 mutant of CFTR or empty vector alone produced the same effect as expression of WT-CFTR on VSOR Cl⁻ currents in C127 cells. A, representative records before and after a hypotonic challenge during application of alternating pulses of ±40 mV or step pulses from −100 to +100 mV in 20 mV-increments (a-d, expended traces on the right) in C127 (upper panel), C127/BPV (second panel), C127/CFTR (third panel) or C127/ΔF508 cells (bottom panel). B, mean Cl⁻ current densities (bar: s.c.m.) recorded at ± 40 mV in C127 (n=9, −30.6±3.6 pA pF⁻¹ at −40 mV), C127/BPV (n=7, −116±12.4 pA pF⁻¹ at −40 mV), C127/CFTR (n=7, −126.6±7.6 pA pF⁻¹ at −40 mV) and C127/ΔF508 (n=7, −111.1±14.0 pA pF⁻¹ at −40 mV) cells. C, I-V relationships for the mean current densities (bar: s.c.m.) in C127 (n=9, open circles), C127/BPV (n=7, filled circles), C127/CFTR (n=7, filled triangles) and C127/ΔF508 (n=7, filled squares) cells.
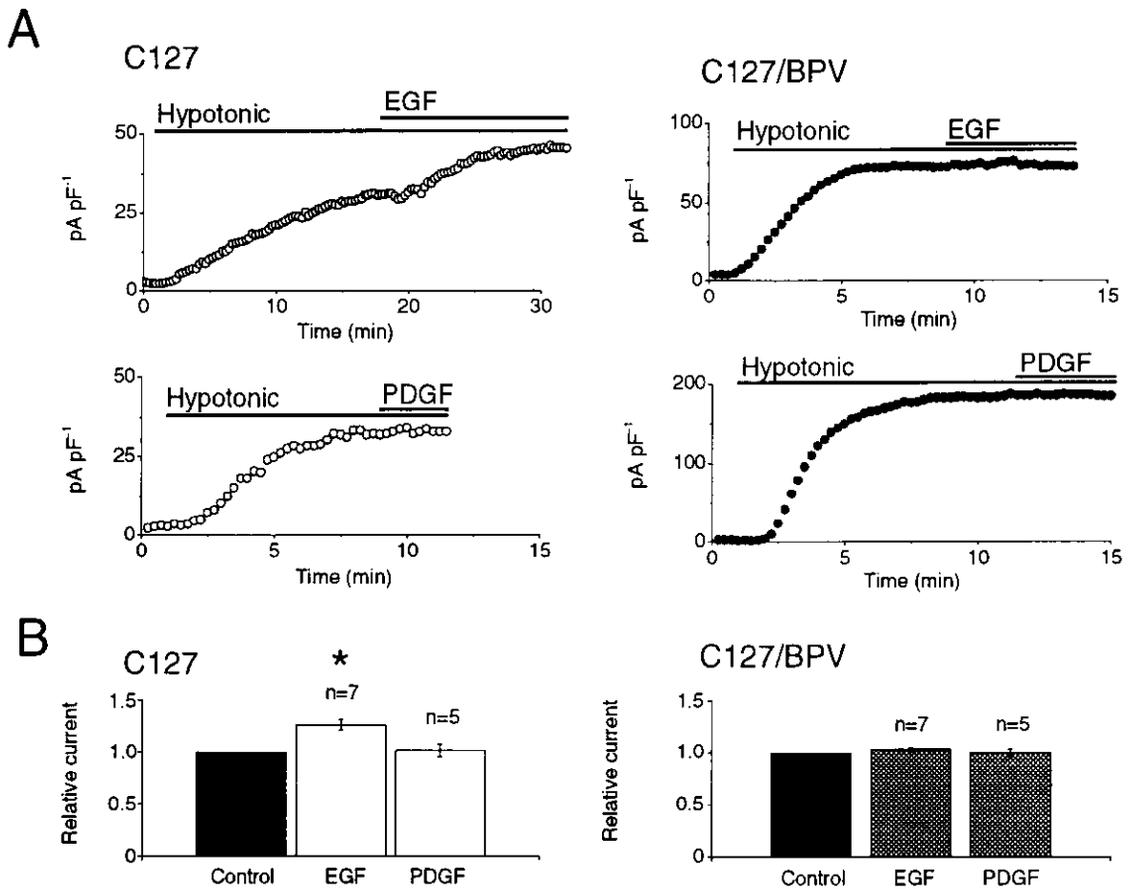
Fig. 14. Effects of growth factors on the VSOR Cl⁻ currents in C127 and C127/BPV cells. *A*, Effect of EGF (50 ng/ml) and PDGF (20 ng/ml) on the steady-state VSOR Cl⁻ currents recorded at +40 mV in C127 and C127/BPV cells. *B*, Relative VSOR Cl⁻ currents before and after application of growth factors. Asterisk, $p<0.05$.
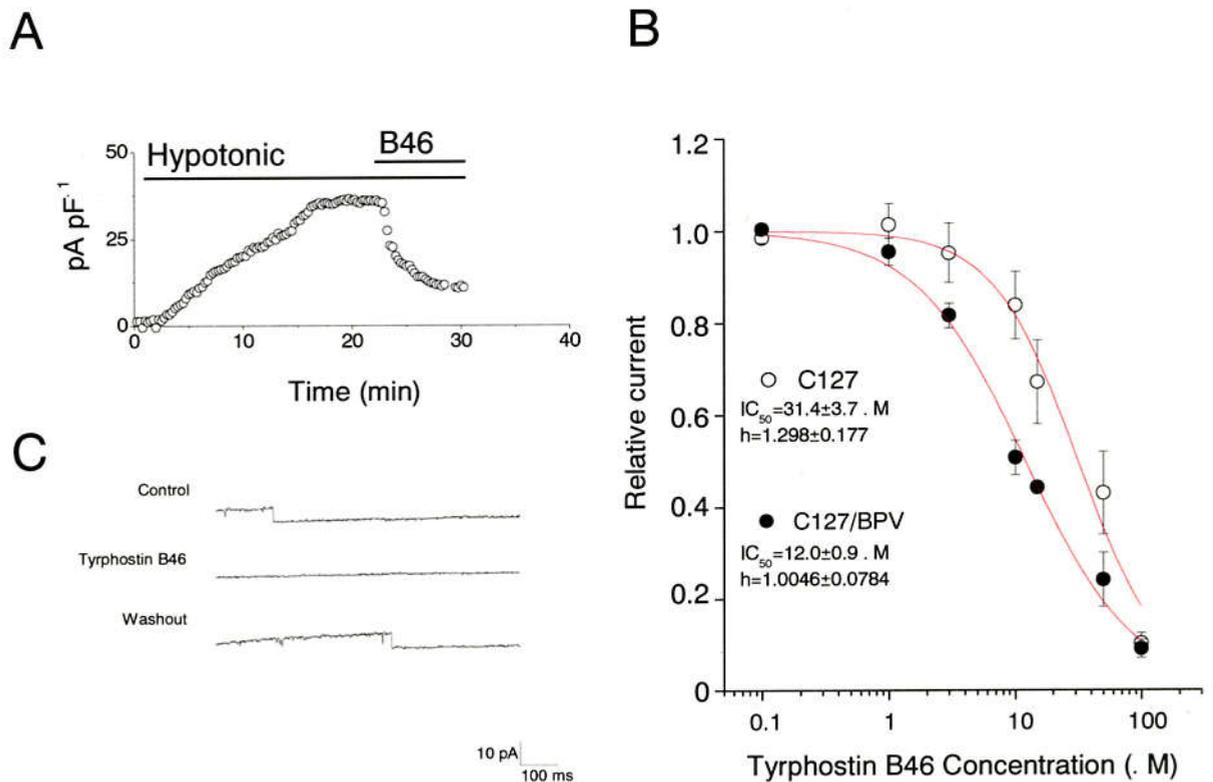
Fig. 15. Effects of EGF tyrosine kinase inhibitor tyrphostin B46 on VSOR Cl⁻ currents in C127 cells. *A*, Effect of tyrphostin B46 (50 . M), a relatively specific inhibitor of EGFR. *C*, VSOR single channel reversibly shut down in a reversible manner upon treatment with tyrphostin B46 (50 . M). *B*, Dose-dependent inhibition of VSOR Cl⁻ currents by tyrphostin B46 in C127 and C127/BPV cells. Tyrphostin was applied after VSOR chloride currents reached the steady-state level. Dose dependency in C127 (filled circles, *n*=4-5) and C127/BPV cells (open squares, *n*=4-5) was fitted using Logistic non-linear curve fit. The IC_{50} values and Hill coefficients (*h*) are given in the figure. *C*, VSOR single channel reversibly shuts down upon treatment with tyrphostin B46 (50 . M).
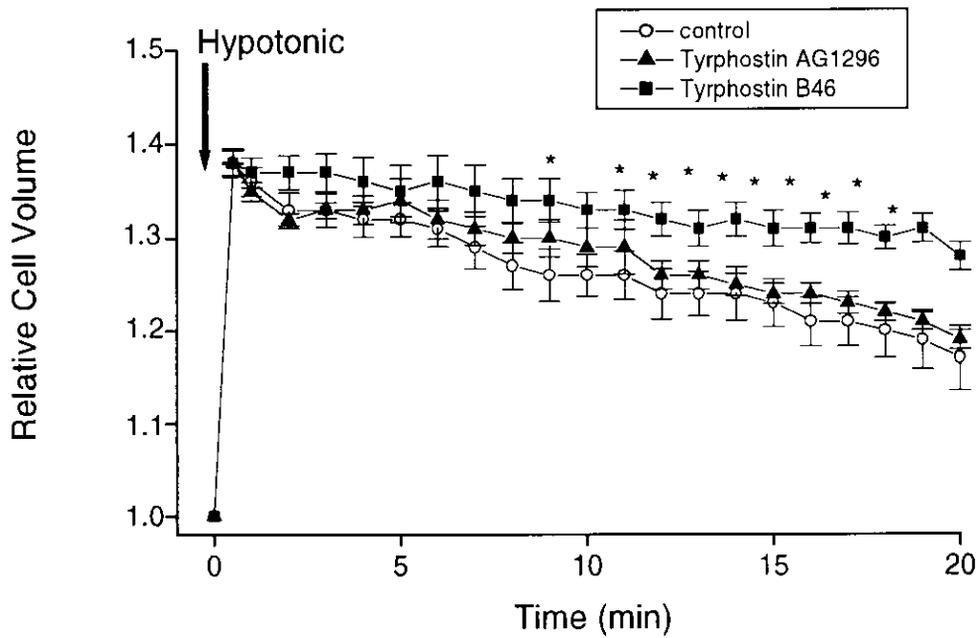
Fig. 16. Volume regulation of C127/BPV cells after osmotic swelling in the absence (open circles) and presence of 50 μM tyrphostin AG1296 (filled triangles) or tyrphostin B46 (filled squares). A hypotonic challenge was applied at time 0. Cell volume was normalized to that before a hypotonic challenge. Each data represents the mean of 5-7 observations. Bar: s.e.m. At asterisks, the mean relative cell volume with tyrphostin B46 was significantly different, but that with tyrphostin AG1296 was not significantly different from the control.
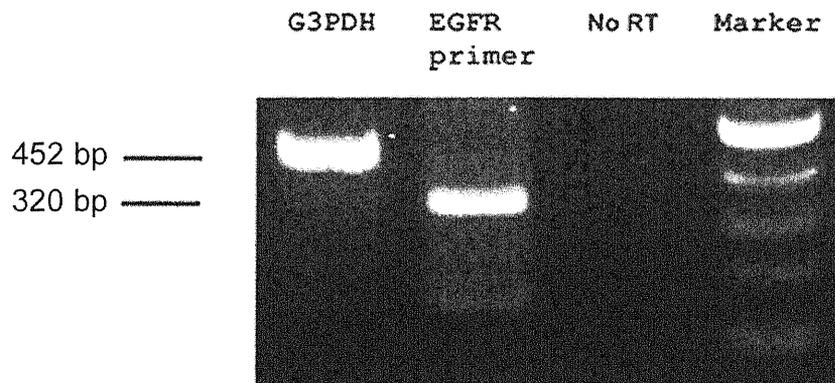
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PCR product: 73 cgtgacttggcagccaggaatgtactggtgaagacaccacagcatgtcaagatcacagat 132

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PCR product: 313 gatggaa 319

mEGFR: 2975 gatggaa 2981

Fig. 17. Expression of EGF receptor mRNA in C127 cells confirmed by RT-PCR. Upper panel: Gel analysis of the EGFR-specific (predicted to be 320 bp) and G3PDH-specific (452 bp) PCR products from C127 cells. Lower panel: Nucleotide sequence of a subcloned PCR product from C127 cells aligned with the sequence of the mouse EGFR (mEGFR).
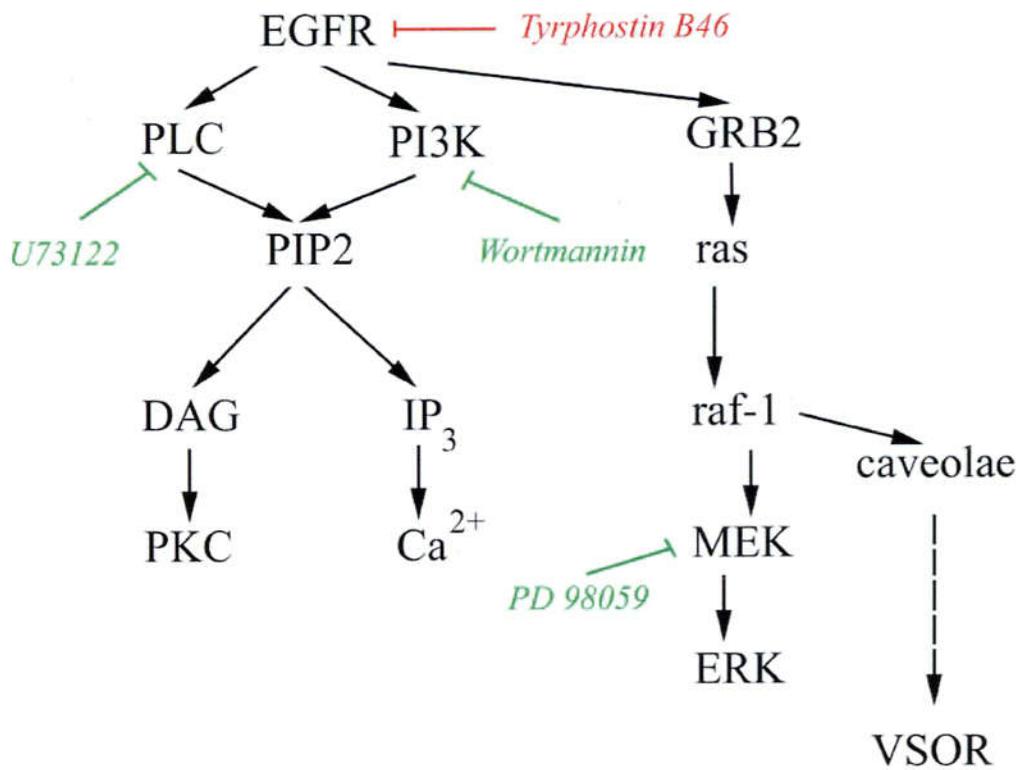
Fig.18. EGF receptor-signaling pathway and inhibitors. *Green*, EGFR-signaling pathway inhibitors which did not inhibit VSOR Cl⁻ currents. *Red*, an EGFR phosphorylation inhibitor which significantly inhibited VSOR Cl⁻ currents.
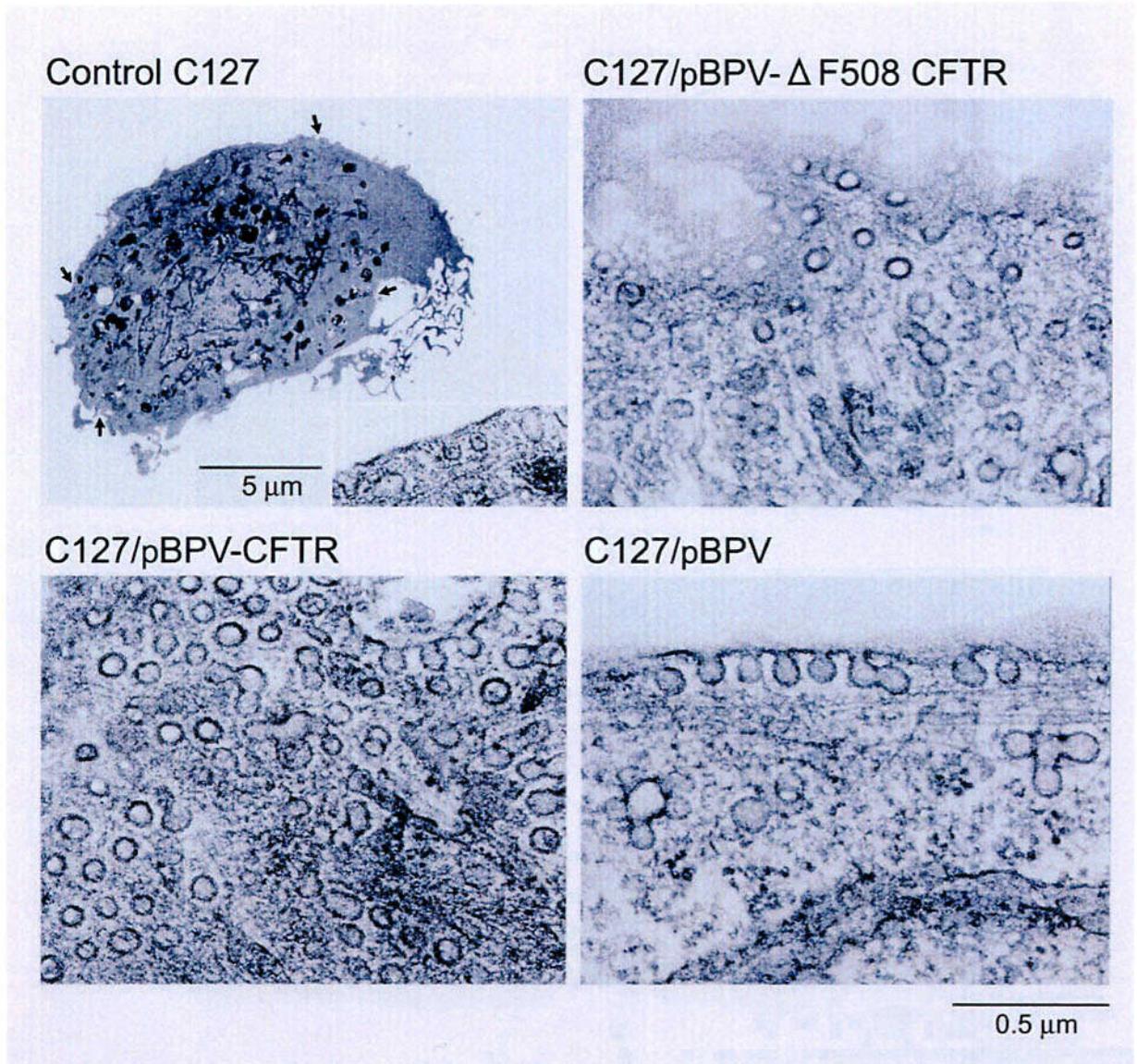
Fig. 19. The number of caveolae was increased by BPV-transfection in C127 cells. Transmission electron microscope studies showed that control C127 cells (*A*) contain much less amount of caveolae (Inset) in their surface membrane compared to BPV-transfected C127/CFTR (*B*), C127/. F508 (*C*), and C127/pBPV (*D*: Mock) cells.