Hyperexpression of recombinant CFTR in heterologous cells alters its physiological properties

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Mohammad-Panah, Raha, Sophie Demolonbe, David Riochet, Veronique Leblais, Gildas Loussouarn, Helene Pollard, Isabelle Barô, and Denis Escande. Hyperexpression of recombinant CFTR in heterologous cells alters its physiological properties. Am. J. Physiol. 274 (Cell Physiol. 43): C310–C318, 1998.—We investigated whether high levels of expression of the cystic fibrosis transmembrane conductance regulator (CFTR) would alter the functional properties of newly synthesized recombinant proteins. COS-7, CFPAC-1, and A549 cells were intranuclearly injected with a Simian virus 40-driven pCE-CFTR plasmid and assayed for halide permeability using the 6-methoxy-N-(3-sulfopropyl)quinolinium fluorescent probe. With increasing numbers of microinjected pCE-CFTR copies, the baseline permeability to halide dose dependently increased, and the response to adenosine 3',5'-cyclic monophosphate (cAMP) stimulation decreased. In cells hyperexpressing CFTR, the high level of halide permeability was reduced when a cell metabolism poisoning cocktail was applied to decrease intracellular ATP and, inversely, was increased by orthovanadate. In CFPAC-1 cells investigated with the patch-clamp technique, CFTR hyperexpression led to a time-independent nonrectifying chloride current that was not sensitive to cAMP stimulation. CFPAC-1 cells hyperexpressing CFTR exhibited no outward rectifying chloride current nor inward rectifying potassium current either spontaneously or under cAMP stimulation. We conclude that hyperexpression of recombinant CFTR proteins modifies their properties inasmuch as 1) CFTR channels are permanently activated and not susceptible to cAMP regulation and 2) they lose their capacity to regulate heterologous ionic channels.

Cystic fibrosis transmembrane conductance regulator; cystic fibrosis; expression; patch clamp; CFPAC-1 cells; A549 cells; epithelial cells

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is caused by mutations in a gene encoding a 1,480-amino acid membrane protein (25), the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR belongs to the ATP binding cassette (ABC) superfamily of ATP-linked transporters (16) and is directly involved in epithelial Cl⁻ secretion, since it is an anionic channel (5) regulated by intracellular ATP and protein kinase A-dependent phosphorylation. The CFTR protein is also a regulator of other ionic channels implicated in water and salt epithelial secretion such as the outward-rectifying Cl⁻ channels implicated in water and salt epithelial secretion (ORCC; see Ref. 11), the amiloride-sensitive Na⁺ channels (33), and the inward rectifying epithelial K⁺ channels (21). The major consequence of the most common deletion of phenylalanine 508 of the CFTR gene is that the mutated protein is abnormally processed and trafficked within affected epithelial cells so that the gene product is virtually absent from the cell membrane (17). Pharmacological treatments targeted to activate directly the CFTR protein would thus be inefficient in the vast majority of CF patients, and the most promising route to control the CF disease is presently gene therapy (27).

One preeminent problem for CF gene therapy is low in vivo efficiency (<0.1% of transduced epithelial cells using an adenovirus vector, as assessed by in situ hybridization in the Knowles et al. (18) protocol). A partial solution to this problem could be to hyperexpress wild-type CFTR transgene in the limited number of successfully transduced cells with the hope to obtain amplification of functional correction (so-called "by-stander" effect) through Cl⁻ movement via gap junction from noncorrected cells into corrected adjacent cells for secretion (6, 7). To this end, high-strength promoters are usually chosen to drive wild-type CFTR cDNA in recombinant gene therapy constructs. However, in the physiological setting, the number of CFTR copies per respiratory epithelial cell is low (500–5000 channel proteins/cell; see Ref. 13), and one may wonder whether hyperexpression of wild-type CFTR impacts its physiological properties. The present study was designed to gain further information on this important issue. We have microinjected into the nucleus of CFTR-deficient cells various concentrations of a Simian virus 40 (SV40)-driven plasmid encoding wild-type CFTR, and we have confirmed dose-effect relationships. The present data show that CFTR hyperexpression profoundly affects its regulation.

METHODS

Cell cultures. COS-7 (an African green monkey kidney-derived cell line) and A549 cells (an alveolar type II epithelium-like cell line) were provided by the American Type Culture Collection (Rockville, MD). COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; all from GIBCO, Paisley, Scotland) at 37°C in a humidified 5% CO₂-95% air incubator and were regularly subcultured by enzymatic treatment with a solution of 0.25% trypsin 1‰ EDTA in a Ca²⁺- and Mg²⁺-free phosphate buffer solution (GIBCO). A549 cells were cultured in Ham's F-12 medium as modified by M. E. Kaighn and supplemented with 10% fetal calf serum. Pancreatic epithelial CFPAC-1 cells isolated from a ΔF508/ΔF508 patient with CF (29) were
cultured as previously reported (26). The CFPAC-PLJ 6-CFTR clone, a kind gift from Dr. R. A. Frizzell (University of Pittsburgh, Pittsburgh, PA; see Ref. 9), was obtained by stably transfecting CFPAC-1 cells with a retroviral vector that contained the cDNA encoding wild-type CFTR gene. The cells were microinjected with plasmids at day 1 after plating on glass coverslips for 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) experiments or on coated plastic petri dishes (Nunc; InterMed Nunc, Roskilde, Denmark) for patch-clamp experiments. In this procedure, the Eppendorf ECET microinjector 5246 system, the ECET micromanipulator 5171 system, and a Nikon Diaphot inverted microscope were used. Nuclear microinjection was performed with the Z (depth) limit option using a 0.3-s injection duration and 40–60 hPa injection pressure. Injection femtotips (internal diameter 0.5 ± 0.2 µm) were borosilicate glass capillaries. Plasmids were diluted at a final concentration ranging from 5 to 350 µg/ml in an injection buffer made of (in mM) 50 N-2-hydroxyethylpipеразин-N’-2-этилсулфоуксусный кислота (HEPES), 50 NaOH, and 40 NaCl, pH 7.4. Fluorescein isothiocyanate (FITC)-labeled dextran (330,000) was also added to the injection medium. To stabilize successfully microinjected cells, immediately before the injection, the injection mixture containing the cDNA sample was centrifuged at 13,000 revolutions/min for 20 min to pellet the particular material. Using radiolabeled technetium-99 as a probe, we estimated that the nuclear volume so injected was ≈10−12 liters. The number of injected cDNA copies was thus estimated to 10,000 at a plasmid concentration of 100 µg/ml. A plasmid construct, pECE-CFTR (a kind gift from Dr. Pascale Fanen, Institut National de la Santé et de la Recherche Méicale (INSERM), Hôpital Henri Mondor, Créteil, France), in which the full-length DNA encoding wild-type CFTR is placed under the control of a SV40 promoter and origin of replication was used for most experiments. SV40 large T antigen is a viral oncoprotein that transactivates viral promoters and induces synthesis in quiescent cells. Large T antigen-expressing cells such as COS-7 cells support a high degree of replication of transfected plasmids containing an SV40 origin of replication. The same construct (pECE; see Ref. 12; a gift from Dr. E. Clauer, Collège de France, Paris, France) lacking the CFTR insert was used for control experiments. Additional control experiments were also performed with a pCE plasmid encoding the sulfonyleurea receptor (pCE-SUR; see Ref. 1; a kind gift from Dr. Lydia Aguilar-Bryan, Baylor College of Medicine, Houston, TX) and with a pSG5H-EBCR plasmid encoding an epithelial basolateral Cl− regulator (see Ref. 35; a kind gift from Dr. Marcel A. Van Kuijck, University of Nijmegen, The Netherlands). In preliminary experiments, we used a reporter β-galactosidase expression vector (pRSV-LacZ; a gift from Dr. Pierre Leh, INSERM, Hôpital Robert Debré, Paris, France). To reveal exogenous β-galactosidase activity, cells were fixed for 15 min with 0.5% formaldehyde and then analyzed histochemically using 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-Gal). SPQ fluorescence assay. Cells placed on glass coverslips were loaded with the intracellular dye SPQ (Molecular Probes) by incubation in Ca2+-free hypotonic (50% dilution) medium containing 10 mM SPQ at 37°C for either 6 min (COS-7) or 10 min (CFPAC-1 and AS49). The coverslip was mounted on the stage of a Nikon Diaphot inverted microscope equipped for fluorescence and illuminated at 360 nm. The emitted light was collected at 456 ± 33 nm by a high-resolution image intensifier coupled to a video camera (Extended ISIS camera system; Photonic Science). The camera was connected to a digital image processing board controlled by FLUO software (Limstar, France). Single-cell fluorescence intensity was measured from digital image processing and displayed against time. Fluorescence intensity was standardized according to F = (F − F0)/F0 × 100, where F is fluorescence and F0 is the fluorescence intensity measured in the presence of I− (36). The membrane permeability to halides was determined as the rate of SPQ dequenching upon perfusion with nitrates. At least three successive data points were collected immediately after the NO3−-containing medium was applied and then fitted using a linear regression analysis. The slope of the relation so measured reflected the membrane permeability to halide. The control Tyrode solution for SPQ experiments contained (in mM) 145 NaCl, 4 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, and 5 glucose, pH adjusted to 7.4 with NaOH. I− and NO3− media were identical to the control Tyrode solution except that I− or NO3− replaced Cl− as the dominant extracellular anion. All extracellular medium used also contained 10 μM bumetanide to inhibit the Cl−-cation cotransporter. Control washout experiments showed that, under our experimental conditions, intracellular SPQ fluorescence declined by only 10.5 ± 2.1% (n = 18) for a period of time as long as 2 h.

Whole cell patch-clamp recordings. Whole cell currents with either the nystatin permeabilized-patch or the ruptured-patch arrangements of the patch-clamp technique were recorded as previously described (3). A petri dish containing cells was placed on the stage of an inverted microscope and continuously superfused with the standard extracellular solution. Patch pipettes with a tip resistance 2.5–5 MΩ were electrically connected to a patch-clamp amplifier (axopatch 200A; Axon Instruments, Foster City, CA). Stimulation, data recording, and analysis were performed by a software made by Gérard Sadoc (distributed by DIPSI Industrie, Asnière, France) through an analog-to-digital converter (Tecmar TM100 Labmaster; Scientific Solution, Solon, OH). Depolarizing voltage ramps were applied at a frequency of 0.2 Hz from −80 to +60 mV (depolarization rate: 46.7 mV/s; holding potential: −60 mV). In experiments aimed to record the ORCC, the membrane voltage was stepped in sequence from a holding potential of −60 to −100 mV for 150 ms, then back to −60 mV for another 150 ms, and then to +60 mV for 500 ms. Current-voltage relationships were constructed with voltage steps applied for 500 ms every 2 s from −60 mV to various potentials between −100 and +60 mV. A micropipette system allowed local application and rapid change of the different experimental solutions warmed at 35°C. The standard Cl−-containing extracellular solution was similar in composition to that used for SPQ experiments. In experiments targeted to record pure Cl− currents, Na+ and K+ were substituted for Cs+ in the extracellular medium; the pipette solution was made of (in mM) 74.5 CsCl, 70.5 aspartic acid, 5 HEPES, 1 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid or ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0–5 MgATP, and 5 glucose, pH 7.2 with CsOH. In experiments targeted to record K+ currents, the extracellular medium contained (in mM) 145 tris(hydroxymethyl)aminomethane (Tris), 2 K2SO4, 1 CaSO4, 1 MgSO4, and 5 glucose, pH 7.4 with H2SO4. For ruptured-patch recordings, the pipette solution contained (in mM) 5 Tris, 72.5 K2SO4, 1 EGTA, 0–5 MgATP, and 5 glucose, pH 7.2 with H2SO4. For experiments performed at 25°C, the extracellular solution was supplemented with 60 mM mannitol to compensate osmotic imbalance caused by the use of nystatin (3).
Drugs. Intracellular adenosine 3',5'-cyclic monophosphate (cAMP) was increased with a mixture made of 10 μM forskolin plus 400 μM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (both from Sigma Chemical, St. Louis, MO). On occasion, 100 μM 3-isobutyl-1-methylxanthine (Sigma) was added to the cAMP stimulating cocktail. Diphenoxyamine-2-carboxylic acid (DPC; 500 μM; Fluka Chemical) was used as a CFTR channel blocker (31). Sodium orthovanadate (Na3VO4; LC Laboratories, Wolum, MA) was used at a final concentration of 1 mM. With the aim to decrease intracellular ATP, we used a cell-poisoning cocktail made of sodium cyanide (2 mM; Aldrich Chemical), 2,4-dinitrophenol (0.1 mM; Sigma), carbonyl cyanide-m-chlorophenyl-hydrazone (5 μM; Sigma), iodoacetic acid (5 μM; Aldrich), and α-2-deoxy-d-glucose (5 mM; Sigma). Drugs were dissolved in dimethyl sulfoxide so that the final concentration of the solvent was 1%.

Statistics. Patch-clamp and SPQ measurements are presented as means ± SE. Statistical significance of the observed effects was assessed by means of the standard t-test.

RESULTS

Intranuclear injection of reporter gene and kinetics of CFTR expression. Using bacterial LacZ as a reporter gene, we first evaluated the efficacy of our intranuclear injection procedure. Injected cells were identified by FITC-dextran fluorescence predominating either in the nucleus or in the cytoplasm. At a pRSV-LacZ plasmid concentration of 100 μg/mL, 73.8 ± 8.1% (mean ± SE; n = 151; 9 assays) of intranuclearly injected COS-7 cells showed blue nuclear staining with X-Gal revelation performed 24 h postinjection. In the CFPAC-1 cell line, the percentage of intranuclearly injected cells demonstrating β-galactosidase expression was 78.7 ± 7.9% (n = 73; 7 assays) with a pRSV-LacZ concentration of 100 μg/mL. As previously reported by others (32), cells microinjected in the cytoplasm with β-galactosidase expression plasmid demonstrated no transgene expression (n = 194 for COS-7 and n = 130 for CFPAC-1).

One of the well-identified advantages of injecting plasmids intranuclearly is that expression of the transgene is fast (32). To gain further insight into expression delay under our experimental conditions, CFPAC-1 cells were first loaded with SPQ, and a cell under microscope observation was injected intranuclearly at time 0 with the pECE-CFTR expression plasmid at 200 μg/mL. The extracellular medium was then switched sequentially between nitrate (filled horizontal bars) and iodide (open horizontal bars) media. cAMP enhancing cocktail was present throughout. Inset: membrane permeability to halide (p expressed in min⁻¹) determined as the initial rate of dequenching in the three solutions (circles, cells injected with pECE-CFTR; crosses, cells injected with pECE vector alone) and plotted against time postinjection.

CFTR hyperexpression in COS-7, CFPAC-1, and A549 cells. We then varied the concentration of the injected pECE-CFTR plasmid and measured corresponding membrane permeability to halide using the SPQ assay. When COS-7 cells were injected with a low concentration of the pECE-CFTR plasmid (i.e., <5 μg/mL), the membrane permeability under baseline was not significantly different from cells injected with the pECE vector alone (Figs. 2A and 3A). As expected, the permeability to halide greatly increased upon application of the cAMP enhancing cocktail (Figs. 2A and 3A). By contrast, COS-7 cells injected with a plasmid concentration >5 μg/mL exhibited an increased membrane permeability under baseline and were less responsive to cAMP stimulation (Figs. 2, A and D, and 3A). In Fig. 2D, the membrane permeability under cAMP stimulation expressed relative to the membrane permeability under baseline was determined in COS-7 cells injected with various concentrations of pECE-CFTR and was plotted as a function of the membrane permeability under baseline. This relation demonstrates that the
more COS-7 cells were permeable to halide under baseline conditions the less responsive they were to cAMP stimulation. Typically, COS-7 cells injected with 200 µg/ml pECE-CFTR exhibited a 40-fold increased baseline permeability that did not increase further with cAMP (Figs. 2, A and D, and 3A). Interestingly, the baseline permeability in COS-7 cells hyperexpressing CFTR exceeded the cAMP-stimulated permeability determined in cells injected with a lower plasmid concentration. Comparable findings were also obtained in CFPAC-1 cells and in A549 cells, although in these cells, the membrane permeability under baseline increased by 10.2 ± 0.3 fold.

Fig. 2. Overexpression and hyperexpression of CFTR channels in COS-7, CFPAC-1, and A549 cells. Relative SPQ fluorescence is plotted against time. A-C: open horizontal bars indicate iodide-containing solution, whereas filled horizontal bars indicate nitrate-containing solution. Filled inverted triangles indicate the time at which the cAMP-enhancing cocktail was applied. A: COS-7 cells injected with the pECE plasmid alone (continuous line) or with 5 µg/ml (filled circles), 100 µg/ml (open circles), or 200 µg/ml (open circles) pECE-CFTR. B: CFPAC-1 cells injected with the pECE plasmid alone (continuous line) or with 100 µg/ml (filled circles), 200 µg/ml (open circles), or 350 µg/ml (open circles) pECE-CFTR. C: A549 cells injected with the pECE plasmid alone (continuous line) or with 100 µg/ml (filled circles) or 350 µg/ml (open circles) pECE-CFTR. D: response to cAMP stimulation determined as the ratio of the membrane permeability under cAMP stimulation (p<sub>cAMP</sub>) in the presence of the cAMP enhancing cocktail to the membrane permeability under baseline (p<sub>baseline</sub>) and plotted as a function of p<sub>baseline</sub> (in min<sup>-1</sup>). Each data point was obtained from different individual COS-7 cells injected with various plasmid concentrations ranging from 5 to 350 µg/ml. Continuous line is a hyperbolic fit.

Fig. 3. Plasmid concentration dependence of baseline (open bars) and cAMP-stimulated (filled bars) permeability to halide (p in min<sup>-1</sup>) in COS-7 (A), CFPAC-1 (B), and A549 (C) cell lines. C, noninjected control cells; pECE, cells injected with the pECE plasmid alone (100 µg/ml); pECE-CFTR, cells injected with various plasmid concentrations ranging from 5 to 350 µg/ml. Data are means ± SE with the number of experiments (n) between 17 and 113. Y-axis for COS-7 and CFPAC-1 cells is discontinuous. Statistical significance vs. control cells injected with pECE alone for baseline permeability (open bars) or vs. cells before cAMP stimulation for cAMP-stimulated permeability: *P < 0.05, **P < 0.01, and ***P < 0.001. Absence of asterisks denotes non-significant differences.
increased only at the highest pECE-CFTR concentration tested (i.e., >200 µg/ml; Figs. 2, B and C, and 3). In CFPAC-1 and in A549 cells injected with 350 µg/ml, the membrane permeability under baseline increased ~10- and 7-fold, respectively. We never observed such behavior in cells injected with the pECE plasmid alone (n = 77 for COS-7; n = 12 in CFPAC-1; n = 15 in A549) or in cells injected with cDNA encoding for other ABC proteins such as pECE-SUR (350 µg/ml; n = 27 for COS-7 and n = 33 for CFPAC-1) or pSG5HN-EBCR (350 µg/ml; n = 38 for COS-7 and n = 19 for CFPAC-1).

This set of experiments suggested that cells injected with a low pECE-CFTR plasmid concentration expressed recombinant Cl⁻-CFTR channels that were closed under basal conditions and opened in response to cAMP-mediated phosphorylation, whereas cells injected with a high pECE-CFTR plasmid concentration possessed permanently opened recombinant Cl⁻ channels that were not susceptible to cAMP-dependent protein kinase (PKA) phosphorylation. The former were defined as overexpressing cells, whereas the latter were defined as hyperexpressing cells.

Pharmacological modulation of hyperexpressed CFTR conductance in COS-7 cells. We next explored the pharmacology of hyperexpressed recombinant CFTR channels using the SPQ assay. The sensitivity of hyperexpressed CFTR channels to DPC (500 µM) was similar to that of overexpressed CFTR protein (Fig. 4). The effects of vanadate stimulation on hyperexpressed CFTR channels were also explored. Vanadate substituted for inorganic phosphate at the ATP-binding fold to stabilize CFTR channels in an open state (4). As shown in Fig. 5, vanadate increased baseline membrane permeability in COS-7 cells hyperexpressing CFTR. This effect was not reversible upon washout of the drug. Finally, we altered the cell metabolism with the objective to explore the sensitivity of hyperexpressed channels to decreased intracellular ATP. Cells were submitted to a metabolism poisoning cocktail containing cyanide (Fig. 6). Upon perfusion with the poisoning cocktail, the membrane permeability under baseline decreased, suggesting that hyperexpressed CFTR channels retained their sensitivity to intracellular ATP.

Patch-clamp recordings of hyperexpressed CFTR current. Patch-clamp experiments performed in 350 µg/ml pECE-CFTR-injected CFPAC-1 or COS-7 cells confirmed that a high-amplitude time-dependent Cl⁻ current was yet available in the absence of cAMP stimulation (Fig. 7A) and did not increase further upon stimulation with forskolin. On average, the current amplitude at +60 mV was 28.6 ± 10.2 pA/pF (n = 6) in CFPAC-1 cells injected with 350 µg/ml pECE-CFTR but only 3.5 ± 0.6 pA/pF (n = 15; P < 0.001) in CFPAC-1 cells injected with the pECE vector alone.

Our next goal was to investigate whether hyperexpressed CFTR proteins would retain their property to regulate other ionic channels. These experiments were performed in CFPAC-1 cells. In a first series of experiments, we used the CFPAC-PLJ 6-CFTR clone, which stably expresses a low level of wild-type CFTR protein. Under experimental conditions that suppressed contaminating K⁺ currents, the average background current was 3.6 ± 0.5 pA/pF at +60 mV (n = 20) in CFPAC-PLJ 6-CFTR cells, not different from parental CFPAC-1 cells (2.9 ± 0.4 pA/pF at +60 mV; n = 11). cAMP stimulation activated a Cl⁻ conductance in 24 out of 58 CFPAC-PLJ 6-CFTR cells (mean current amplitude: 14.5 ± 2.8 pA/pF at +60 mV; n = 24) but not in parental CFPAC-1 cells (Fig. 7B; n = 43). The current activated by cAMP in CFPAC-PLJ 6-CFTR cells was further investigated with a voltage step protocol as shown in Fig. 7, B-E. Close examination of the current traces revealed that the Cl⁻ current activated by cAMP in CFPAC-PLJ 6-CFTR was the sum of 1) a time-
independent Cl\textsuperscript{−} current lacking rectification that supposedly corresponded to CFTR current and 2) a time-dependent current that slowly activated upon depolarization and exhibited outward rectification (Fig. 7C). We identified the latter as a Cl\textsuperscript{−} current flowing through ORCC. CFTR and ORCC currents were also activated by 1) extracellular ATP in CFPAC-1 cells transduced or not with the wild-type CFTR gene (n = 10; data not shown) and 2) cAMP stimulation in CFPAC-1 cells injected with a low pECE-CFTR concentration (Fig. 7D). These latter experiments were conducted in 30% Cl\textsuperscript{−}-containing solutions (70% gluconate) to improve the quality of voltage control in injected cells exhibiting large Cl\textsuperscript{−} currents. Twenty-seven cells were recorded under 30% Cl\textsuperscript{−}: 10 out of 27 exhibited cAMP-activated Cl\textsuperscript{−} currents compatible with CFTR plus ORCC coactivation as shown in Fig. 7D, 7 out of 27 showed Cl\textsuperscript{−} currents compatible with CFTR activation alone, and 2 out of 27 with ORCC alone and 8 out of 27 did not respond to cAMP stimulation. Thus CFPAC-1 cells expressing wild-type CFTR possess ORCC channels that activate in response to cAMP stimulation, as previously demonstrated by Schwiebert et al. (31) in 9HTE\textsuperscript{−} epithelial cells. Under K\textsuperscript{+}-free conditions, CFPAC-1 cells injected with 350 µg/ml pECE-CFTR never exhibited ORCC current neither at baseline nor under cAMP stimulation (n = 25 cells; Fig. 7E). These investigations were conducted either in the ruptured-patch configuration with 5 mM MgATP in the pipette solution or, alternatively, in the permeabilized-patch configuration. Additional experiments were also conducted in low 10 or 30% Cl\textsuperscript{−} solution (28 cells) or in iodide-containing solution (5 cells). These experiments were all unsuccessful at identifying ORCC current.

In a study also conducted in CFPAC-1 cells (21), we have previously identified an inwardly rectifying K\textsuperscript{+} current that responds to cAMP stimulation in the exclusive presence of functional CFTR channels. Figure 8 shows that the cAMP-activatable inwardly rectifying K\textsuperscript{+} current was recorded in CFPAC-1-PLJ6-CFTR cells and also in parental CFPAC-1 cells injected with a low CFTR plasmid concentration (n = 7). Under Cl\textsuperscript{−}-free conditions, no inwardly rectifying K\textsuperscript{+} current was identified either at baseline or under cAMP stimulation (16 cells; ruptured-patch or permeabilized-patch configurations; Fig. 8C). On average, in injected CFPAC-1 cells bathed in Cl\textsuperscript{−}-depleted solutions, the background K\textsuperscript{+} current was 0.9 ± 0.1 pA/pF at +10 mV (n = 16), not significantly different from noninjected or pECE-injected cells (0.8 ± 0.1 pA/pF at +10 mV; n = 13). From these data, we concluded that hyperexpressed CFTR channels lose their property to regulate heterologous Cl\textsuperscript{−} or K\textsuperscript{+} channels.
DISCUSSION

In the present study, the use of direct intranuclear cDNA injection provided us with the opportunity to vary the amount of recombinant CFTR protein produced in a given cell and to evaluate the potentially deleterious consequences of hyperexpression on CFTR physiology. Our data suggest that CFTR hyperexpression alters the basic properties of the recombinant protein in such a way that CFTR channels 1) are permanently opened in the absence of cAMP stimulation and exhibit reduced sensitivity to phosphorylation by PKA; 2) have a conserved sensitivity to intracellular ATP regulation; and 3) lose their property to regulate heterologous ionic channels. Using radiolabeled technetium, we estimated that the intranuclearly injected volume was in the order of 10⁻²⁻² liters, in accordance with previous calculations made by others (22). Therefore, the number of copies injected in the nucleus was in the range of 500–35,000 for plasmid concentrations between 5 and 350 µg/ml. In comparison, common lipotransfection methods bring about 100,000 copies in the cytoplasm (19). Hyperexpression as reported here has previously been observed by Stutts et al. (34) in NIH/3T3 fibroblasts stably transfected with a retroviral construct carrying wild-type CFTR: five fibroblastic clones were identified for expressing the CFTR protein using Western blot analysis. Among these, three clones (nos. 3, 5, and 10) demonstrated a higher level of CFTR expression by densitometric estimates of CFTR-specific immunostaining. These clones exhibited depolarized membrane potential at baseline, a consistent background Cl⁻ conductance, and an altered sensitivity to cAMP stimulation. Typically, in clone 5, which maintained the most elevated basal current mediated by CFTR, there was no response of the whole cell current to forskolin stimulation.

The reason why hyperexpression produces permanently opened CFTR channels remains obscure. Honoré et al. (15) previously reported that different amounts of cRNAs encoding Kv1.3 K⁺ channels produced biophysically and pharmacologically distinguishable expressions of K⁺ channel activity in Xenopus oocytes; with a low amount of cRNA, Kv1.3 channels exhibited time- and voltage-dependent inactivation and were fully blocked by 10 nM charybdotoxin, whereas, with a high amount of cRNA, recombinant Kv1.3 channels did not inactivate and were hardly blocked by charybdotoxin. Injection of cRNA at intermediate concentrations induced K⁺ channels with properties corresponding to a mixture of biophysical and pharmacological properties observed for the inactivating and noninactivating K⁺ channels obtained at low and high cRNA concentra-

Fig. 7. Whole cell recordings of Cl⁻ currents under K⁺-free conditions in CFPAC-1 cells. A: from left to right, superimposed current traces of the background current in a CFPAC-1 cell injected with pECE vector alone (CFTR⁻), in a CFPAC-PLJ6-CFTR cell [CFTR+ (PLJ6)], in a cell injected with 100 µg/ml pECE-CFTR (CFTR+), and in a cell injected with 350 µg/ml pECE-CFTR (CFTR+++). Protocol consisted of voltage steps, 20 mV in increment, applied from -60 mV to various voltages between -100 and +60 mV. Vertical bar: 200 pA; horizontal bar: 100 ms. B-E: current-voltage curves of Cl⁻ currents induced by cAMP and obtained by digital subtraction. Insets show corresponding current traces (vertical bar: 200 pA; horizontal bar: 100 ms). Same voltage protocol as in A. B: parental CFPAC-1 cell (CFTR⁻). C: CFPAC-PLJ6-CFTR cell [CFTR+ (PLJ6)]. D: CFPAC-1 cell overexpressing CFTR and injected with 100 µg/ml pECE-CFTR. E: CFPAC-1 cell hyperexpressing CFTR and injected with 350 µg/ml pECE-CFTR. In D and E, external and intrapipette medium contained only 30% Cl⁻ (70% gluconate) to improve voltage control.

Fig. 8. Inwardly rectifying K⁺ currents in CFPAC-1 cells. cAMP-induced K⁺ currents obtained by digital subtraction and recorded with a ramp protocol under Cl⁻-free conditions. Recordings are from a CFPAC-1 cell injected with pECE alone (A), a CFPAC-PLJ6-CFTR cell (B), a CFPAC-1 cell overexpressing CFTR and injected with 100 µg/ml pECE-CFTR (C), and a CFPAC-1 cell hyperexpressing CFTR and injected with 350 µg/ml pECE-CFTR (D).
tions, respectively. Inactivating and noninactivating $K^+$ channels were also observed in IM-9 human B lymphocytes stably transfected with Kv1.3 cDNA (15). Our study suggests that a comparable behavior also exists with CFTR channels. Most recently, Larsen et al. (20) reported that CFTR $Cl^-$ channel clusters spontaneously entered a mode of high open probability as a result of cooperative interaction between neighboring channel proteins. Previously, it was suggested that CFTR channels are tonically blocked by the R domain plugging the channel pore and that phosphorylation on serines by protein kinase A electrostatically repels the R domain, allowing the passage of $Cl^-$ (8). Expression of a variant protein in which the R domain was deleted resulted in the appearance of $Cl^-$ channels that were active in the absence of added cAMP and showed only a small additional response to cAMP (24). One interpretation of our results is that hyperexpression produces huge clusters in which the high density of CFTR proteins creates charge-charge interactions between the different R domains. Interaction may push these domains away from the channel pore and maintain the channels in the open state in the absence of cAMP stimulation. However, our results do not provide any experimental support to this interpretation, and alternative explanations should also be considered, including cytoskeleton interactions (14) or synthesis of an immature form of the CFTR protein. Of interest is the possibility that a portion of CFTR channels are constitutively active at every level of CFTR expression. In cells overexpressing CFTR, the proportion of constitutively active CFTR channels is too small to be detectable with the assay we use. In cells hyperexpressing CFTR, the number of constitutively active CFTR channels increases and is yet detectable with the SPQ assay. Obviously, further experiments are needed to get some insight into the mechanism leading to permanently opened CFTR channels during hyperexpression.

Potentially the farthest reaching implication of our results is that hyperexpression of CFTR in targeted epithelial cells may affect CFTR functions that are physiologically important in the organ targeted. For example, this may concern the capacity of CFTR to regulate other epithelial ionic channels and thereby to coordinate water and salt secretion. The mechanism that governs regulation by CFTR of other ionic channels is still unknown. One proposed hypothesis has been that the CFTR channel pore drives ATP outside the cell membrane to activate neighboring channels through an autocrine mechanism (30), although the capacity of CFTR channels to conduct ATP has most recently been disputed (23). Our own results also oppose the ATP hypothesis, since one may expect that permanently opened CFTR channels as induced by hyperexpression should also drive ATP to activate the ORCC channels even in the absence of cAMP stimulation. Schiavi et al. (28) previously showed that high levels of wild-type CFTR expression lead to cell growth perturbation and can be deleterious to the normal function of the cells. Our study extends this concept to the molecular physiology of the CFTR protein itself. We anticipate that the level of CFTR expression obtained with direct intranuclear plasmid injection may be greater than that achieved in vitro with more conventional transfection techniques (maybe with the exception of retroviral infection; see Ref. 34). Therefore, the deleterious consequences of CFTR hyperexpression as observed with direct intranuclear cDNA injection in vitro may require stronger promoters and/or highly more efficient transfection vectors than currently used in vivo gene therapy. However, with the aim to obtain a bystander effect of CFTR gene transfer, higher-strength promoters are currently developed for clinical use. Our study suggests that hyperexpression and consequent alterations in CFTR molecular physiology may potentially result from this search.

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