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Transfected β3- but Not β2-Adrenergic Receptors Regulate Cystic Fibrosis Transmembrane Conductance Regulator Activity via a New Pathway Involving the Mitogen-Activated Protein Kinases Extracellular Signal-Regulated Kinases

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ABSTRACT

We have shown previously that in a heterologous mammalian expression system A549 cells, β_3-adrenoceptor (β_3-AR) stimulation regulates the activity of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. The present investigation was carried out to determine the signaling pathway involved in this regulation. A549 cells were intranuclearly injected with plasmids encoding human CFTR and β_3-AR. CFTR activity was functionally assessed by microcytofluorimetry. The application of 1 μM 4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-1 hydrochloride (CGP-12177), a β_3-AR agonist, produced a CFTR activation that was not abolished by protein kinase A inhibitors. In pertussis toxin-pretreated cells, the CFTR activation induced by CGP-12177 was abolished. The overexpression of β-adrenoceptor receptor kinase, an inhibitor of $\beta\gamma$ subunits, abolished the CGP-12177–induced CFTR activation, suggesting the involvement of $\beta\gamma$ subunits of $G_{i/o}$ proteins. The pretreatment of A549 cells with selective inhibitors of either phosphoinositide 3-kinase (PI3K), wortmannin, and 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002), or extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK), 2′-amino-3′-methoxyflavone (PD98059), and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene (U0126), abolished the effects of CGP-12177 on the CFTR activity. Immunohistochemical assays showed that only the cells expressing β_3-AR exhibited MAPK activation in response to CGP-12177. Furthermore, CFTR activity increased in cells pretreated with 10% fetal bovine serum both in A549 cells injected only with CFTR and in T84 cells, which endogenously express CFTR, indicating that CFTR activity can be regulated by the MAPK independently of the β_3-AR stimulation. In conclusion, we have demonstrated that CFTR is regulated through a $G_{i/o}$/PI3K/ERK1/2 MAPK signaling cascade dependently or not on an activation of β_3-ARs. This pathway represents a new regulation for CFTR.

Cystic fibrosis (CF), the most common lethal recessive genetic disorder among white persons, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Collins, 1992). The gene encodes the CFTR protein, a cAMP-regulated Cl^- channel that controls salt and water transport across the epithelium in many tissues. CFTR proteins are expressed in airway epithelia and submucosal glands, sweat glands, gastrointestinal tract, pancreatic bile, and reproductive ducts. Gating of the channel is tightly regulated by phosphorylation of the regulatory domain. The regulatory domain contains multiple phosphorylation sites for cAMP-dependent protein kinase A (PKA) (Cheng et al., 1991). Phosphorylation at most of these sites stimulates Cl^-

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ABBREVIATIONS: CF, cystic fibrosis; β-AR, β-adrenoceptor; β-ARK, β-adrenergic receptor kinase; CFTR, cystic fibrosis transmembrane conductance regulator; CGP-12177, 4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-1 hydrochloride; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PTX, pertussis toxin; SPQ, halide-sensitive fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolium; SR 58611A, (*R*,*S*)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2 hydroethanamide hydrochloride; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; PD98059, 2′-amino-3′-methoxyflavone; ERK1/2, extracellular signal-regulated kinases 1 and 2; Rp-8-Br-cAMPS, Rp-8-bromoadenosine-3′,5′-cyclic monophosphorothiorate; Rp-8-CPT-cAMPS, Rp-8-(chlorophenyl-thio)adenosine-3′,5′-cyclic monophosphorothiorate; CL 316243, disodium 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino)propyl]-1,3-benzodioxole-2,2-dicarboxylate; BRL 37344, (±)-(*R**,*R**)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium.

channel activity (Wilkinson et al., 1997). CFTR also contains protein kinase C (PKC) phosphorylation sites. There is evidence that prior phosphorylation by PKC primes CFTR for subsequent phosphorylation and activation by PKA (Jia et al., 1997; Liedtke and Cole, 1998). The mechanism for this priming effect of PKC is unknown (Kirk, 2000). CFTR can also be activated by cGMP via cGMP-dependent protein kinase (PKG)-mediated phosphorylation in cells expressing the PKGII isoform but not in airway epithelium (Berger et al., 1993) that lack PKGII isoforms (French et al., 1995). Finally, several studies suggest that CFTR might be regulated either directly or indirectly by tyrosine phosphorylation (Dahan et al., 2001).

β-adrenoceptor (β-AR) stimulation is well known to regulate the intracellular cAMP levels. β-AR agonists exert a variety of effects on airway epithelial cells, such as an increase in ciliary beat frequency (Sanderson and Dirksen, 1989) and an activation of ion transport by opening apical ion channels like CFTR (Bennett, 2002; Salathe, 2002). In epithelial cells, cAMP also activated CFTR (Gadsby and Nairn, 1999), but no data are available concerning an elevation of cAMP by β_2-AR stimulation in those cells. However, in *Xenopus laevis* oocytes, the β_2-AR stimulation produced an activation of adenylyl cyclase that increased the concentration of intracellular cAMP, leading to an activation of CFTR channel gating (Wotta et al., 1997). Our group has demonstrated in a recombinant system, a human lung epithelial-derived cell line (A549), that CFTR is regulated by β_3-AR stimulation produced either by isoproterenol (a nonselective β-AR agonist) in the presence of nadolol (a β_1, β_2-AR antagonist), SR 58611A (a preferential β_3-AR agonist), or CGP-12177 (a partial β_3-AR agonist) (Leblais et al., 1999). This CFTR activation induced by β_3-AR stimulation was not prevented by PKA inhibitors, ruling out the involvement of a cAMP/PKA pathway in this response (Leblais et al., 1999). Thus, the present investigation was carried out to characterize the signaling pathway involved in the regulation of CFTR by β_3-AR in A549 cells. Using pharmacological and biochemical approaches, we demonstrated the involvement of a new signaling pathway in the regulation of CFTR: $G_{i/o}$ protein/phosphoinositide 3 kinase (PI3K)/ERK1/2 mitogen-activated protein kinase (MAPK).

Materials and Methods

Cell Culture. The human lung epithelial-derived cell line A549 and the human colonic carcinoma cell line T84 were provided by the American Type Culture Collection (Manassas, VA). A549 cells were cultured as reported previously (Mohammad-Panah et al., 1998). T84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) (Invitrogen, Paisley, UK) maintained in a humidified incubator (95% air/5% CO_2) at 37°C and passaged weekly.

Plasmids. Transgene cDNAs were subcloned into $pcDNA_3$ mammalian expression vector under the control of a cytomegalovirus promoter. The $pcDNA_3$-CFTR plasmid (a gift from J. Ricardo, Lisbon, Portugal) encoded the wild-type human CFTR protein, and the $pcDNA_3$-β_2 and $pcDNA_3$-β_3A plasmids (gifts from D. Langin, Toulouse, France) encoded human β_2-AR and A isoform of the human β_3-AR, respectively. The $pcDNA_3$-β-adrenoceptor receptor kinase (β-ARK) plasmid (a gift from R. J. Lefkowitz, Durham, NC) encoded the C-terminal region of β-ARK.

Intranuclear Injection of Plasmids. Cells were microinjected with plasmids (30 μg/ml for human CFTR, 0.1 μg/ml for human β_3-AR, and/or 0.3 μg/ml for human β_2-AR) 1 day after plating on glass coverslips (Nunclon; NUNC A/S, Roskilde, Denmark). Our protocol to intranuclearly microinject individual cells using the Eppendorf ECET microinjector 5246 system and the ECET micromanipulator 5171 system has been reported in detail elsewhere (Mohammad-Panah et al., 1998). Plasmids were diluted in an injection buffer composed of 50 mM HEPES, 50 mM NaOH, and 40 mM NaCl, pH 7.4. Fluorescein isothiocyanate-labeled dextran (0.5%) was added to the injection medium to visualize successfully microinjected cells.

SPQ Fluorescence Assay. The Cl^- channel activity of CFTR was assessed using the halide-sensitive fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolium (SPQ; Molecular Probes, Leiden, Netherlands) as described previously (Mohammad-Panah et al., 1998). Twenty-four hours after injection, cells were loaded with intracellular SPQ dye by incubation in Ca^{2+}-free hypotonic (50% dilution) medium containing 10 mM SPQ at 37°C for 15 min. The coverslips were mounted on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan) 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, Roberts-Bridge, UK) connected to a digital-image processing board controlled by FLUO software (Imstar, Paris, France). Cells were maintained at 37°C and continuously superfused with an extracellular solution containing 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 5 mM glucose, pH 7.4. A microperfusion system allowed local application and rapid change of the different experimental media. I^-- and NO_3^--containing media were identical with the extracellular solution except that I^- and NO_3^- replaced Cl^- as the dominant extracellular anions. All extracellular media also contained 10 μM bumetamide to inhibit the $Cl^-/Na^+/K^+$ cotransporter.

Single-cell fluorescence intensity was measured from digital-image processing and displayed against time. Fluorescence intensity was standardized according to the equation $F = (F - Fo)/Fo \times 100$, where F is the relative fluorescence and Fo is the fluorescence intensity measured in the presence of I^-. 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 NO_3^--containing medium application and then fitted using a linear regression analysis. The slope of the straight line reflected the membrane permeability to halides and was used as an index of CFTR activity.

Immunohistochemical Assays. After injection, cells were placed for 12 h in a medium containing 10% fetal bovine serum (FBS). Then, cells were placed in a medium without FBS for an additional 12 h to reduce basal levels of phosphorylation. After aspiration of the culture medium, cells were treated by adding fresh medium without FBS, containing or not 1 μM CGP-12177 for 5 min at 37°C. Cells were washed with phosphate-buffered saline and fixed successively in 3% paraformaldehyde for 30 min at 4°C and 100% methanol for 10 min at −20°C. After washing with phosphate-buffered saline, the fixed cells were incubated with primary monoclonal antibody raised against human β_3-AR (a gift from GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK) for 3 h at room temperature. This antiserum was revealed with peroxidase-conjugated second serum. After the control of the efficiency of the β_3-AR staining procedure under microscope, a second staining was performed with the phospho-p44/42 MAPK antiserum (Thr202/Tyr204; Cell Signaling Technology Inc., Beverly, MA) overnight at 4°C and revealed with alkaline phosphatase-conjugated second serum.

Drugs. Intracellular cAMP was increased with a mixture containing 10 μM forskolin plus 100 μM 3-isobutyl-1-methylxanthine (both from Sigma-Aldrich, Saint Quentin Fallavier, France). CGP-12177 was purchased from Sigma-RBI (Clermont-Ferrand, France); salbutamol, (−)-isoproterenol, wortmannin, and bumetamide were from Sigma-Aldrich; and pertussis toxin (PTX), PD98059, LY294002, and

U0126 were from Calbiochem (Meudon, France). The two PKA inhibitors, Rp-8-bromoadenosine-3′,5′-cyclic monophosphorothiorate (Rp-8-Br-cAMPS) and Rp-8-(chlorophenyl-thio)adenosine-3′,5′-cyclic monophosphorothiorate (Rp-8-CPT-cAMPS) were obtained from Biolog (Bremen, Germany). For SPQ experiments, drugs were dissolved in dimethyl sulfoxide (Sigma-Aldrich) so that final concentration of the solvent was less than 0.1%.

Statistical Analysis. Data are expressed as the means ± S.E. of n experiments. The statistical significance of a drug effect versus baseline was assessed using a one-way analysis of variance completed when appropriate by a Bonferroni test. The significant influence of a pretreatment on a drug effect was assessed by two-way analysis of variance.

Results

CFTR Activation by β_3-AR Stimulation. In A549 cells injected only with the plasmid encoding human CFTR, the application of 10 μM forskolin, a direct activator of adenylate cyclase, increased the p value by 8-fold, whereas the application of 1 μM CGP-12177 did not modify basal CFTR activity (Fig. 1A). In A549 cells coexpressing CFTR and β_3-AR, the CFTR basal permeability to halides (p) was 0.15 ± 0.01 min^{-1}. The application of increasing concentrations (0.01–10 μM) of CGP-12177 (a β_3-AR agonist) produced an increase of p values in a concentration-dependent manner. The maximal effect was obtained at 1 μM. At this concentration, CGP-12177 increased p values by approximately 6-fold after 15 min of perfusion (Fig. 1B). Then, for the following experiments, CGP-12177 was used at 1 μM.

Involvement of $G_{i/o}$ Protein in CFTR Activation Induced by β_3-AR Stimulation. To determine the putative involvement of PKA in the CFTR regulation by β_3-AR stimulation, cells were incubated for 20 min with a mixture of two PKA inhibitors, Rp-8-Br-cAMPS (100 μM) and Rp-8-CPT-cAMPS (100 μM). In such conditions, the effect of CGP-12177 on CFTR activity was not modified (Fig. 2A). To check the efficiency of PKA inhibitors, the effects of isoproterenol, a nonselective β-AR agonist, and salbutamol, a β_2-AR agonist,

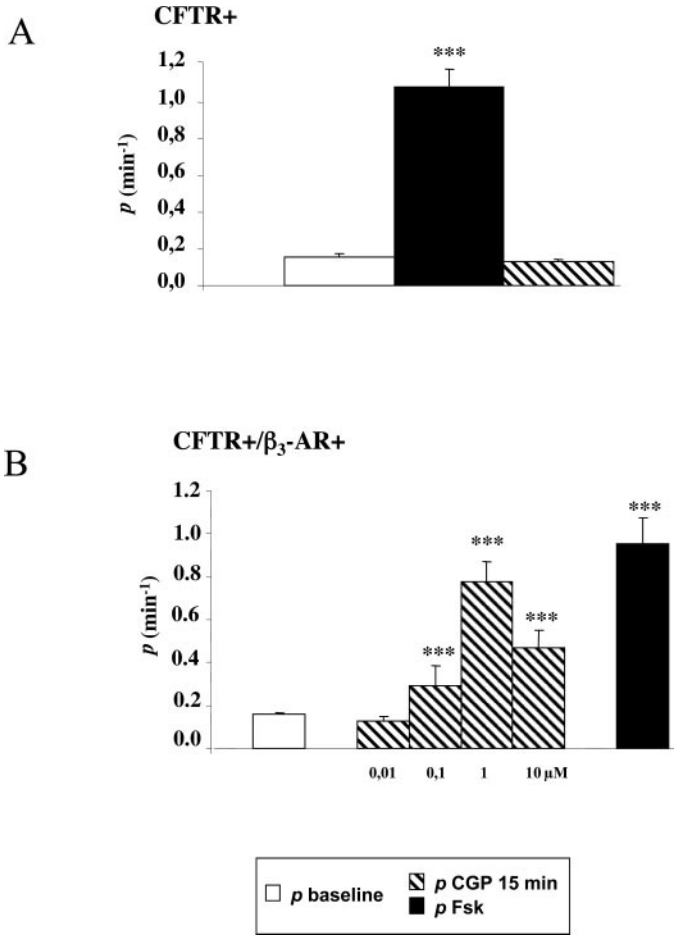

Fig. 1. CFTR regulation by β_3-AR stimulation in A549 cells. A, in cells injected with CFTR (30 μg/ml) cDNA, membrane permeability to halides (p in minutes^{-1}) was measured under baseline (□) and after 15 min of perfusion with 10 μM forskolin (Fsk) or 1 μM CGP-12177 (CGP), a β_3-AR agonist. B, in cells coinjected with CFTR (30 μg/ml) and β_3-AR (0.1 μg/ml) cDNAs, membrane permeability to halides (p in minutes^{-1}) was measured under baseline and during β_3-AR stimulation performed by 15 min of perfusion with different concentrations of CGP-12177. The functional presence of CFTR was confirmed by application of 10 μM forskolin. Data are the means ± S.E. of 35 to 86 cells. ⋆⋆⋆, $p < 0.001$ versus baseline.

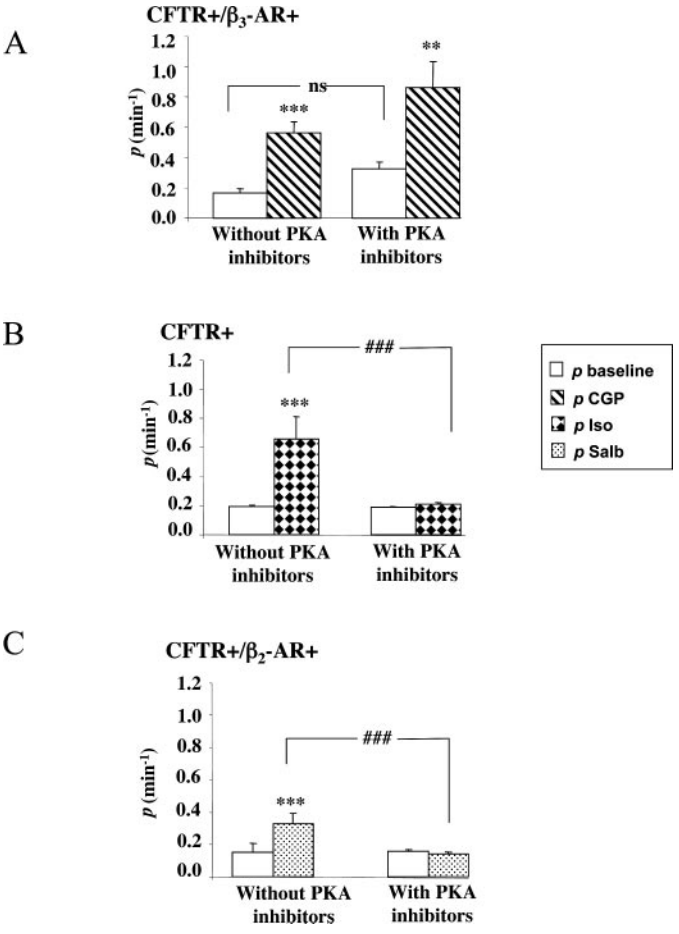

Fig. 2. Effect of PKA inhibitors on CFTR regulation by β-AR stimulation. A, in A549 cells coinjected with CFTR cDNA (30 μg/ml) and β_3-AR cDNA (0.1 μg/ml), membrane permeability to halides (p in minutes^{-1}) was measured under β_3-AR stimulation performed with 1 μM CGP-12177 for 15 min (CGP) in the absence or the presence of two PKA inhibitors (100 μM Rp-8-Br-cAMPS and 100 μM Rp-8-CPT-cAMPS). B, in cells injected with CFTR cDNA alone (30 μg/ml), membrane permeability to halides (p in minutes^{-1}) was measured after 15-min application of 1 μM isoproterenol (Iso) in the absence or the presence of both PKA inhibitors. C, in cells coinjected with CFTR cDNA (30 μg/ml) and β_2-AR cDNA (0.3 μg/ml), membrane permeability to halides (p in minutes^{-1}) was measured under β_2-AR stimulation performed with 1 μM salbutamol for 15 min (Salb) in the absence or the presence of both PKA inhibitors. Data are the means ± S.E. of 25 to 68 cells. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus baseline; ###, $p < 0.001$ versus untreated cells; ns, not significant.

were investigated in the absence and presence of PKA inhibitors (Rp-8-Br-cAMPS and Rp-8-CPT-cAMPS). In A549 cells injected with CFTR alone, isoproterenol through stimulation of the endogenous β_1- and/or β_2-AR stimulation increased significantly the activity of CFTR. This effect was abolished in cells pretreated with PKA inhibitors (Fig. 2B). In cells injected with CFTR and β_2-AR, 10 μM salbutamol increased significantly the CFTR activity. This effect was fully abolished in cells pretreated with PKA inhibitors (Fig. 2C).

The pretreatment with PTX (500 ng/ml) during 36 h did not modify the activation of CFTR induced by forskolin in cells expressing CFTR alone (Fig. 3A). In contrast, in cells coexpressing CFTR and β_3-AR, the response to CGP-12177 was fully abolished after pretreatment with PTX (Fig. 3B), suggesting that CFTR activation by β_3-AR stimulation involved $G_{i/o}$ proteins. To determine the $G_{i/o}$ subunits ($\alpha_{i/o}$ and/or $\beta\gamma$) involved in this effect, we coinjected A549 cells with the plasmid encoding for the C-terminal tail of β-ARK (5 μg/ml), an inhibitor of the $\beta\gamma$ complex (Koch et al., 1994), in addition to plasmids encoding for CFTR and β_3-AR. In such conditions, the CFTR activation induced by CGP-12177 was fully inhibited. In the other hand, the forskolin response was not modified (Fig. 4).

Involvement of the PI3K-MAPK Pathway in CFTR Activation Induced by β_3-AR Stimulation. In cells pretreated with 50 μM wortmannin, a PI3K inhibitor (Gerhardt et al., 1999), for 25 min, CGP-12177 only increased p values by approximately 2-fold (Fig. 5A). After 25-min pretreatment with 10 μM LY294002, a highly selective PI3K inhibitor (Gerhardt et al., 1999), the response to CGP-12177 was fully abolished (Fig. 5B). The effects of CGP-12177 were also abolished in cells deprived of FBS, an activator of MAPK pathway, and pretreated for 24 h with either 20 μM PD98059 (Fig. 6A) or 10 μM U0126 (Fig. 6B), two highly selective ERK1/2 MAPK inhibitors (Gerhardt et al., 1999).

To strengthen the MAPK involvement in CFTR regulation suggested by the present pharmacological studies, a sequential dual immunolabeling method was used. To avoid the activation of MAPK by the serum, the A549 culture medium was replaced by a fresh medium without FBS. Successfully injected cells with β_3-AR were identified by fluorescein isothiocyanate under an incident light fluorescence microscope (Fig. 7). Then, cells were incubated in the absence or the presence of 1 μM CGP-12177 during 5 min. In the first step, incubation with the antibody raised against human β_3-AR was performed. We verified that the noninjected cells did not present any staining, whereas cells injected with β_3-AR were labeled after this procedure (Fig. 7, B and C). In the second step, a second incubation was performed with the phospho-p44/42-MAPK antiserum. No labeling was obtained in wild-type or microinjected cells untreated by CGP-12177. On the other hand, a strong nuclear and perinuclear labeling was obtained with the phospho-p44/42 MAPK antibody only in cells injected with β_3-AR and pretreated with CGP-12177 (Fig. 7, B and C).

Activation of CFTR through the MAPK Pathway Independently of β_3-AR Stimulation. Another set of experiments was performed in A549 cells injected with CFTR

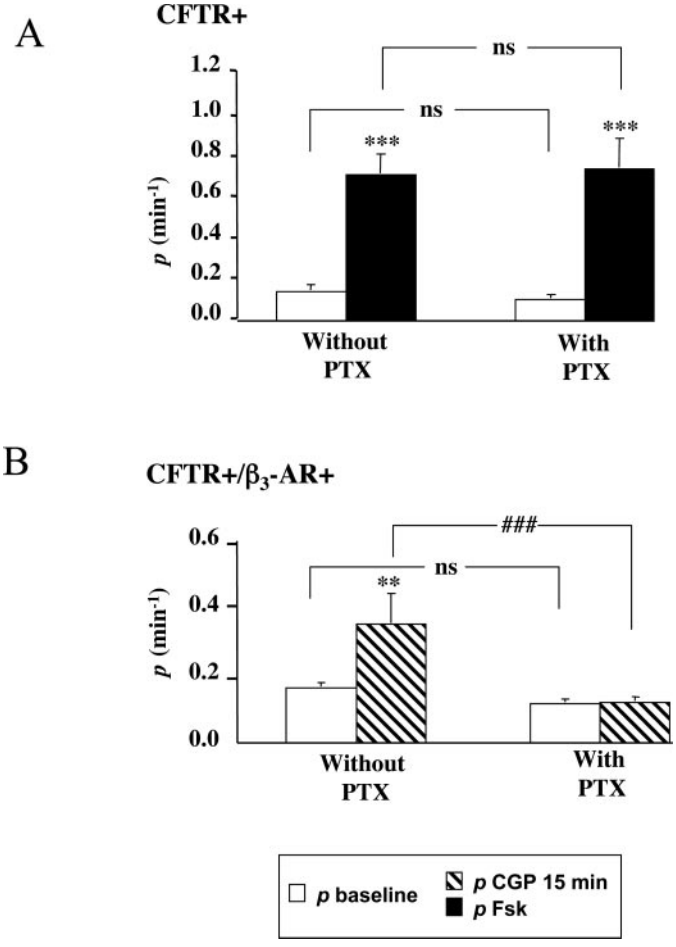

Fig. 3. Effect of PTX pretreatment on CFTR regulation by β_3-AR stimulation in A549 cells. Cells were injected with CFTR cDNA (30 μg/ml) in the absence (A) or presence (B) of β_3-AR cDNA (0.1 μg/ml). p Value was measured under baseline and under application of 10 μM forskolin (Fsk) or during β_3-AR stimulation with 1 μM CGP-12177 for 15 min in the absence or presence of the $G_{i/o}$ inhibition with PTX (500 ng/ml for 36 h). Data are the means ± S.E. of 35 to 39 cells. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $P < 0.001$ versus baseline; ###, $p < 0.01$ versus untreated cells; ns, not significant.

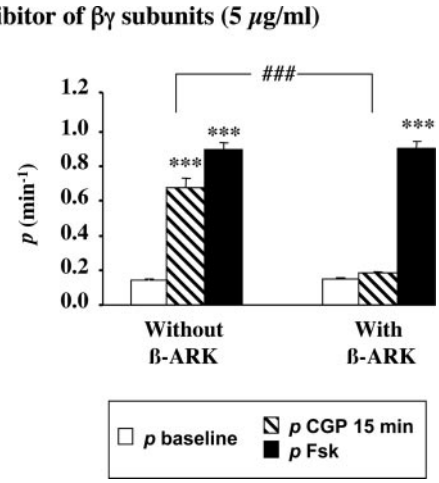

Fig. 4. Effect of β-ARK on CFTR regulation by β_3-AR stimulation in A549 cells. Cells were injected with CFTR (30 μg/ml) and β_3-AR (0.1 μg/ml) cDNAs in the absence or the presence of β-ARK cDNA (5 μg/ml; an inhibitor of the $\beta\gamma$ complex). Membrane permeability to halides (p in minutes^{-1}) was measured during baseline and after β_3-AR stimulation performed with 1 μM CGP-12177 for 15 min (CGP). Direct activation of CFTR was obtained by application of 10 μM forskolin (Fsk). Data are the means ± S.E. of 41 cells. ⋆⋆⋆, $p < 0.001$ versus baseline; ###, $P < 0.01$ versus untreated cells.

alone to determine whether CFTR activation in response to the MAPK pathway recruitment might be observed in the absence of β_3-AR stimulation. A 15-min application of FBS, which is known to be an activator of MAPK pathway (Schramek et al., 1996), increased p values by 5-fold (Fig. 8A). The pretreatment of cells with PD98059 significantly decreased the CFTR activation induced by FBS without modifying the response to forskolin (Fig. 8A). In T84 cells, which endogenously express the CFTR protein (Cohn et al., 1992), the application of FBS increased p values by 3-fold. CFTR activation induced by FBS was also abolished by the pretreatment of T84 cells with PD98059 (Fig. 8B).

Discussion

We have demonstrated previously that β_3-ARs were functionally coupled to the CFTR protein (Leblais et al., 1999). In the present study, using pharmacological and biochemical approaches, we have demonstrated that the regulation of CFTR by β_3-ARs in A549 cells is independent of the cAMP/PKA pathway but involves a $G_{i/o}$ protein/PI3K/ERK1/2 MAPK pathway. This last pathway could also regulate CFTR activity independently of the β_3-AR stimulation.

β_3-AR stimulation induced by CGP-12177 increased CFTR activity in a concentration-dependent manner, with a maximal effect at 1 μM. This increase was not observed in A549 cells only injected with the plasmid encoding CFTR alone, demonstrating that the partial β_3-AR agonist, CGP-12177, activated only β_3-ARs in our recombinant system. This increase of the CFTR activity induced by β_3-AR stimulation was not modified by the pretreatment of A549 cells with PKA inhibitors. In contrast, in cells expressing only CFTR, the increased CFTR activity induced by the stimulation of endogenous β_1- and β_2-ARs by isoproterenol was abolished in PKA inhibitor-pretreated cells. Furthermore, in cells coexpressing CFTR and β_2-AR, salbutamol also produced an activation of CFTR that was abolished by PKA inhibitors. All together, these results demonstrated that 1) the blockade of PKA was efficient in our model, 2) the G−-adenylyl cyclase/cAMP/PKA pathway was functional in A549 cells, and 3) the overexpression of the β_2-AR did not disturb its coupling to cAMP/PKA pathway. Thus, our results demonstrated that β_3-AR did not link to the cAMP/PKA pathway in A549 cells, although this pathway was present and functional.

CFTR activation by the β_3-AR stimulation was abolished by pretreatment with PTX, indicating that PTX-sensitive G proteins, $G_{i/0}$, were involved in this effect. Several studies performed in different cellular types or tissues have already

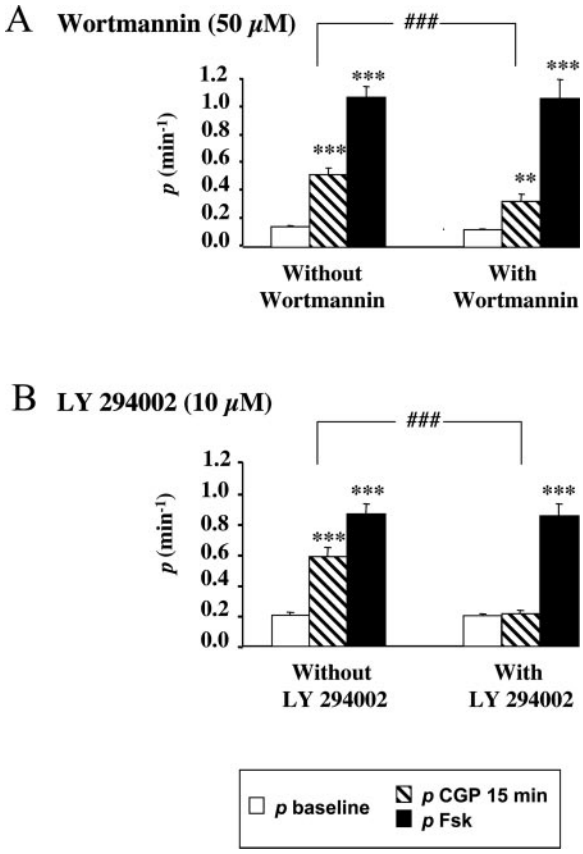

Fig. 5. Effect of PI3K inhibitors on CFTR regulation by β_3-AR stimulation in A549 cells. Cells were injected with CFTR (30 μg/ml) and β_3-AR (0.1 μg/ml) cDNAs. Membrane permeability to halides (p in minutes^{-1}) was measured under baseline and β_3-AR stimulation performed with 1 μM CGP-12177 for 15 min (CGP) in cells pretreated or not with 50 μM wortmannin (A) or 10 μM LY244002 (B), two PI3K inhibitors. The direct activation of CFTR was obtained by application of 10 μM forskolin (Fsk). Data are the means ± S.E. of 25 to 33 cells. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus baseline; ###, $p < 0.001$ versus untreated cells.

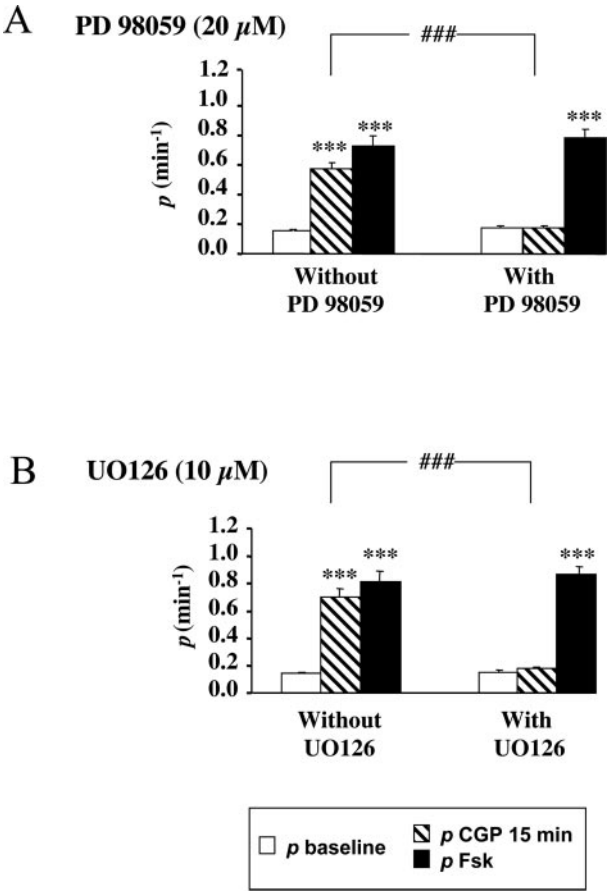

Fig. 6. Effect of MAPK inhibitors on CFTR regulation by β_3-AR stimulation in A549 cells. Cells were injected with CFTR (30 μg/ml) and β_3-AR (0.1 μg/ml) cDNAs. Membrane permeability to halides (p in min^{-1}) was measured under baseline and β_3-AR stimulation performed with 1 μM CGP-12177 for 15 min (CGP) in cells pretreated or not with 20 μM PD98059 (A) or 10 μM U0126 (B), two MAPK inhibitors. Direct activation of CFTR was obtained by application of 10 μM forskolin (Fsk). Data are the means ± S.E. of 30 to 34 cells. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ versus baseline; ###, $p < 0.001$ versus untreated cells.

reported that β_3-ARs could be linked to G_s and/or $G_{i/0}$ proteins. In CHO cells transfected with rat β_3-ARs (Granneman et al., 1991) and murine 3T3-F442A preadipocytes (Feve et al., 1991), β_3-ARs seemed to be coupled to G_s proteins, leading to an increase in intracellular cAMP level. In rat and mice adipocytes, in addition to their coupling to G_s, β_3-ARs coupled to $G_{i/0}$ proteins, which inhibited adenylyl cyclase (Chaudhry et al., 1994; Begin-Heick, 1995). Finally, in some tissues such as human endomyocardial biopsies, β_3-ARs were shown to be exclusively linked to $G_{i/0}$ proteins (Gauthier et al., 1996). The coupling of β_3-ARs to several types of G proteins is not specific to this receptor. Such apparently "promiscuous" coupling has been actually observed with several G protein-coupled receptors, even in native cells (Gudermann et al., 1997). Thus, the β_2-AR has been shown to activate both G_s and $G_{i/0}$ proteins in rat adult cardiac myocytes (Xiao et al., 1995).

Then, to determine whether $\alpha_{i/0}$ subunit and/or $\beta\gamma$ complex of the $G_{i/o}$ protein were involved in CFTR activation induced by the β_3-AR stimulation, A549 cells were coinjected with the plasmid encoding the C-terminal region of β-ARK, an inhibitor of the $\beta\gamma$ complex, in addition to the plasmids encoding CFTR and β_3-ARs. In such conditions, the CFTR activation induced by the β_3-AR stimulation was fully abolished, indicating a total regulation of CFTR by the $\beta\gamma$ complex in our model. It should be noted that several studies reported that CFTR activity could be regulated by the α_i subunit. In cultured human airway epithelial cells, the purified α_i subunit produced an inhibition of CFTR-induced current, probably through an impairment of the CFTR trafficking (Schwiebert et al., 1994; Schreiber et al., 2001). In the presence of exogenous $\beta\gamma$ complex, the inhibition of CFTR-induced current was abolished, probably because of the assembly of the different subunits in an inactive $\alpha\beta\gamma$ complex (Stow et al., 1991).

In the present study, the pretreatment of A549 cells by selective inhibitors of PI3K and ERK1/2 MAPK abolished the CFTR activation induced by β_3-AR stimulation. These pharmacological data were strengthened by the immunohistochemical study, showing a strong nuclear and perinuclear labeling with the phospho-p44/42 MAPK antibody in A549 cells incubated with the β_3-AR agonist. This labeling was absent in wild-type or microinjected cells untreated with the β_3-AR agonist. All together, these results demonstrate that β_3-AR regulates CFTR activity through activation of a $G_{i/o}$ protein/PI3K/MAPK pathway. Our results are in agreement with those of previous studies reporting that β_3-ARs stimulated the MAPK pathway via the activation of $G_{i/0}$ proteins. In CHO/K1 cells overexpressing the human β_3-AR, the β_3-AR was simultaneously coupled to G_s and $G_{i/0}$ proteins, leading to the activation of adenylyl cyclase and MAPK, respectively (Gerhardt et al., 1999). In human embryonic kidney 293 cells expressing human β_3-AR as well as in intact 3T3-F442A adipocytes, CL 316243, a β_3-AR agonist, stimulated the MAPK pathway in a PTX-sensitive G_i-dependent manner rather than in a G_s-dependent manner (Soeder et al., 1999). In contrast, in 3T3-L1 adipocytes, Mizuno and colleagues (2000) proposed that several β_3-AR agonists (BRL 37344, CGP-12177, and SR 58611A) produced an ERK1/2 phosphor-

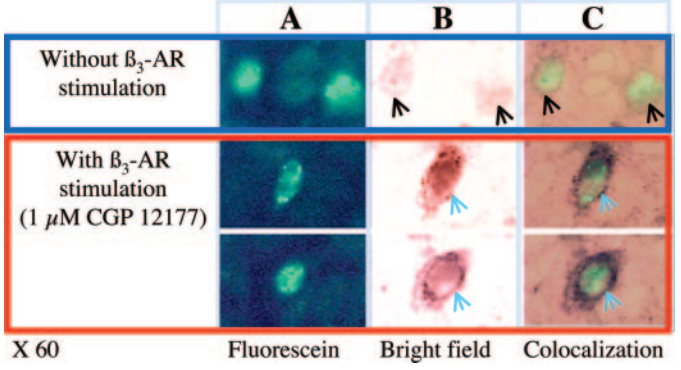

Fig. 7. Phospho-p44/42 MAPK and β_3-AR expression in A549 cells pretreated or not with 1 μM CGP-12177. Cells were injected with β_3-AR cDNA (0.1 μg/ml) and fluorescein isothiocyanate-labeled dextran. A and C, the nuclei of microinjected cells were stained successfully with fluorescein. β_3-AR and phospho-p44/42 MAPK antibodies were revealed with peroxidase and alkaline phosphatase-conjugated second serums, respectively (B and C). On bright-field photomicrographs (×60) performed on cells untreated with CGP-12177, black arrowheads indicate cells labeled with β_3-AR antibody (brown staining in blue panel; B and C). On cells incubated for 5 min with 1 μM CGP-12177, blue arrowheads show cells stained in brown with β_3-AR antibody and strongly labeled in black with phospho-p44/42 MAPK antibody (red panel; B and C).

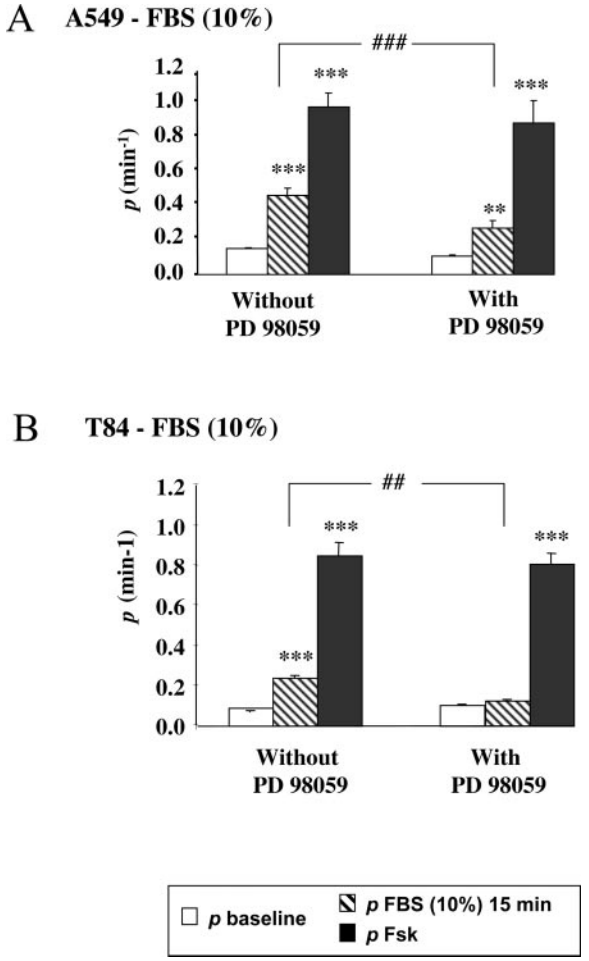

Fig. 8. Activation of CFTR by the MAPK pathway independently of β_3-AR stimulation. A, A549 cells were injected with CFTR cDNA (30 μg/ml) alone. B, T84 cells expressed endogenous CFTR. Membrane permeability to halides (p in min^{-1}) was measured under baseline and after 15-min application of 10% FBS in cells pretreated or not with 20 μM PD98059, a MAPK inhibitor. The direct activation of CFTR was obtained by application of 10 μM forskolin (Fsk). Data are the means ± S.E. of 15 to 88 cells. ★★★, $p < 0.001$ versus baseline; ##, $p < 0.01$; ###, $p < 0.001$ versus untreated cells.

ylation via a pathway involving G_s protein and PKA but not via a G_i protein-dependent pathway. The different coupling of β_3-ARs to G_s and/or G_i according to the studies could result from the diversity of the pharmacological agents used and/or the cell types.

In the present work, we have demonstrated that CFTR regulation by MAPK also occurred independently of the β_3-AR stimulation. This response has been observed both in a recombinant system, A549 cells injected with the plasmid encoding for CFTR, and in native cells, T84, which endogenously express CFTR. To our knowledge, this is the first report relating the regulation of CFTR activity through the MAPK, although an endogenous activation of MAPK has been reported in CF (Ratner et al., 2001). Through the binding on their epithelial receptor sites, adherent bacteria produced increases in intracellular Ca^{2+} concentration, leading to MAPK activation and initiated interleukin-8 production (Ratner et al., 2001). This latter effect contributed to the general proinflammatory medium in CF airways (Heeckeren et al., 1997). However, the consequences of the MAPK activation were not identified.

The present work has been carried out in experimental cell systems and has highlighted a new regulation pathway for CFTR protein, the $G_{i/o}$/PI3K/ERK1/2 MAPK pathway. Further investigations are clearly necessary to determine whether MAPK could modulate CFTR activity in epithelial cells from healthy patients and those with CF. Until now, it has been widely believed that cAMP-dependent phosphorylation of CFTR is the predominant physiological mechanism for activating CFTR in a number of epithelial cells from airways, pancreas, intestines, and sweat glands. However, our results demonstrate that an alternative pathway of CFTR regulation by β-AR stimulation, involving the β_3-AR, exists. These findings might play an important role in airway pathologies in which β_1- and β_2-ARs are decreased, such as in CF (Sharma and Jeffery, 1990).

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