

**Transfected beta3 but not beta2-adrenergic receptors
regulate CFTR activity via a new pathway involving the
mitogen-activated protein kinases-ERK^{\$}**

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Abbreviations: A549, human lung epithelial-derived cell line; 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, foetal bovine serum; FITC, Fluorescein isothiocyanate; FSK, forskolin; MAPK, Mitogen Activated Protein Kinase; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent Protein Kinase; PKC, Protein Kinase C; PKG, cGMP-dependent Protein Kinase; PTX, pertussis toxin; SPQ, halide-sensitive fluorescent probe 6-methoxy-N-(3-sulfopropyl) quinolium; T84, colonic carcinoma cell line; SR 58611A, (RS)-N-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2 hydroethanamide hydrochloride; SR 59230A, 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronapht-1-yl-amino]-2S-2-propanol oxalate.

Abstract

We have previously shown 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 microcytometry. The application of 1 μ M CGP 12177, a β_3 -AR agonist, produced a CFTR activation which was not abolished by protein kinase A inhibitors. In PTX-pretreated cells, the CFTR activation induced by CGP 12177 was abolished. The overexpression of β -ARK, 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 PI3K, wortmannin and LY 294002, or ERK 1/2 MAPK, PD 98059 and 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% foetal 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- ERK 1/2 MAPK signaling cascade dependently or not of an activation of β_3 -ARs. This pathway represents a new regulation for CFTR.

Introduction

Cystic fibrosis (CF), the most common lethal recessive genetic disorder among Caucasians, is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene (Collins et al., 1992). The gene encodes the CFTR protein, a cAMP-regulated chloride (Cl⁻) channel that controls salt and water transport across epithelium in many tissues. CFTR proteins are expressed in airways 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 (R domain). R domain contains multiple phosphorylation sites for cAMP-dependent Protein Kinase (PKA) (Cheng et al., 1991). Phosphorylation at most of these sites stimulates Cl⁻ 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 airways 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 et al., 1999) and an activation of ion transport by opening apical ion channels like CFTR (Salathe, 2002; Bennett, 2002). In epithelial cells, cAMP also activated CFTR (Gadsby and Nairn, 1999), but no data is available concerning an elevation of cAMP by β_2 -AR stimulation in those cells. However, in *xenopus* 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 was regulated by β_3 -AR stimulation produced either by isoproterenol (a non selective β -AR agonist) in the presence of nadolol (a β_1 , β_2 -AR antagonist), or 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 in order 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) - ERK 1/2 Mitogene Activated Protein Kinase (MAPK).

Experimental procedures

Cells 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 (Rockville, MD, USA). A549 cells were cultured as previously reported (Mohammad-Pannah 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 UI/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Paisley, UK)), maintained in a humidified incubator (95% air - 5% CO₂) at 37°C and passaged weekly.

Plasmids. Transgene cDNAs were subcloned into pcDNA₃ mammalian expression vector under the control of a cytomegalovirus promoter. The pcDNA₃-CFTR plasmid (a gift from J. Ricardo, Lisbon, Portugal) encoded the wild-type human CFTR protein, the pcDNA₃-β₂ and pcDNA₃-β_{3A} plasmids (gifts from D. Langin, Toulouse, France) encoded human β₂-AR and A isoform of the human β₃-AR, respectively. The pcDNA₃-β-Adrenoceptor Receptor Kinase (β-ARK) plasmid (a gift from RJ. Lefkowitz, Durham, North Carolina, USA) encoded the C-terminal region of β-ARK.

Intranuclear injection of plasmids. Cells were micro-injected with plasmids (30 µg/ml for human CFTR, 0.1 µg/ml for human β₃-AR and or 0.3 µg/ml for human β₂-AR) at 1 day after plating on glass coverslips (Nunc; InterMed Nunc, 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 details elsewhere (Mohammad-Pannah et al., 1998). Plasmids were diluted in an injection buffer made of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 50 mM NaOH, and 40 mM NaCl, pH 7.4. Fluorescein isothiocyanate (FITC)-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-Pannah et al., 1998). 24 h post-injection, cells were loaded with intracellular SPQ dye by incubation in Ca²⁺-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 (Nikon Diaphot, Japon) 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, France). Cells were maintained at 37°C and continuously superfused with an extracellular solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES and 5 mM glucose, pH 7.4. A microperfusion system allowed local application and rapid change of the different experimental mediums. Iodure (I⁻) and nitrate (NO₃⁻) containing mediums were identical to the extracellular solution except that I⁻ and NO₃⁻ replaced Cl⁻ as the dominant extracellular anions. All extracellular mediums also contained 10 μM bumetamide in order to inhibit the Cl⁻/Na⁺/K⁺ co-transporter. Single-cell fluorescence intensity was measured from digital image processing and displayed against time. Fluorescence intensity was standardized according to the equation $F = (F - F_0) / F_0 \times 100$, where F is the relative fluorescence and F₀ 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 NO₃⁻ containing medium application, and then fitted using a linear regression analysis. The slope of the straight line reflected the membrane permeability to halides, was used as an index of CFTR activity.

Immunohistochemical assays. After injection, cells were placed for 12 h in a medium containing 10% foetal bovine serum (FBS). Then, cells were placed in a medium without FBS for additional 12 h in order to reduce basal level of phosphorylation. After aspiration of the culture medium, cells were treated by adding fresh medium without FBS, containing or not 1 μ M CGP 12177 for 5 min at 37°C. Cells were washed with PBS and fixed successively in 3% paraformaldehyde for 30 min at 4°C and 100% methanol for 10 min at -20°C. After washing with PBS, the fixed cells were incubated with primary monoclonal antibody raised against human β_3 -AR (a gift from GlaxoBeecham, Harlow, UK) for 3 h at room temperature. This antiserum was revealed with peroxylase 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, USA) overnight at 4°C and revealed with alkaline phosphatase conjugated second serum.

Drugs. Intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) was increased with a mixture containing 10 μ M forskolin plus 100 μ M 3-isobutyl-1-methylxanthine (both from Sigma, Saint Quentin Fallavier, France). CGP 12177 (4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-1) was purchased from RBI (Clermont-Ferrand, France), salbutamol, (-)-isoproterenol, wortmannin and bumetamide from Sigma, pertussis toxin (PTX), PD 98059, LY 294002 and U0126 from Calbiochem (Meudon, France). The two PKA inhibitors, Rp-8-Br-cAMPS (Rp-8-bromoadenosine-3',5'-cyclic monophosphorothiorate) and Rp-8-CPT-cAMPS (Rp-8-(chlorophenyl-thio)adenosine-3',5'-cyclic monophosphorothiorate) were obtained from Biolog (Bremen, Germany). For SPQ experiments, drugs were dissolved in dimethylsulfoxide (DMSO; Sigma) so that final concentration of the solvent was less than 0.1%.

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

Results

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

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 μ M) and Rp-8-CPT-cAMPS (100 μ M). 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 non-selective β -AR agonist, and salbutamol, a β_2 -AR agonist, 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, salbutamol at 10 μ M 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). By contrast, in cells co-

expressing CFTR and β_3 -AR, the response to CGP 12177 was fully abolished after pretreatment by PTX (Fig.3B), suggesting that CFTR activation by β_3 -AR stimulation involved $G_{i/o}$ proteins. In order to determine the $G_{i/o}$ subunits ($\alpha_{i/o}$ and/or $\beta\gamma$) involved in this effect, we co-injected A549 cells with the plasmid encoding for the C-terminal tail of β -ARK (5 μ g/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. Conversely, 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 μ M wortmannin, a PI3K inhibitor (Gerhardt et al., 1999) during 25 min, CGP 12177 only increased p values by around 2-fold (Fig.5A). After 25 min pretreatment with 10 μ M LY 294002, 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 during 24 h with either 20 μ M PD 98059 (Fig.6A) or 10 μ M UO126 (Fig.6B), two highly selective ERK 1/2 MAPK inhibitors (Gerhardt et al., 1999).

To strengthen the MAPK involvement in CFTR regulation, suggested by the present pharmacological studies, a sequential dual immuno-labeling 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 FITC under an incident light fluorescence microscope (Fig.7). Then, cells were incubated in the absence or the presence of 1 μ M CGP 12177 during 5 min. In a first step, an incubation with the antibody raised against human β_3 -AR was performed. We verified that the non-injected cells did not present any staining whereas cells injected with β_3 -AR were labeled after this

procedure (Fig.7B, C). In a second step, a second incubation was performed with the phospho-p44/42-MAPK antiserum. No labeling was obtained in wildtype or microinjected cells untreated by CGP 12177. Conversely, 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.7B, 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 alone in order 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 value by 5-fold, (Fig.8A). The pretreatment of cells with PD 98059 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 value by 3-fold. CFTR activation induced by FBS was also abolished by the pretreatment of T84 cells with PD 98059 (Fig.8B).

Discussion

We have previously demonstrated 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 - ERK 1/2 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 μ M. 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. By 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 co-expressing CFTR and β_2 -AR, salbutamol also produced an activation of CFTR which was abolished by PKA inhibitors. All together, these results demonstrated that (i) the blockade of PKA was efficient in our model, (ii) the Gs-adenylyl cyclase-cAMP-PKA pathway was functional in A549 cells and (iii) 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 the pretreatment with PTX, indicating that PTX sensitive-G proteins, $G_{i/o}$, were involved in this effect. Several studies

performed in different cellular types or tissues have already reported that β_3 -ARs could be linked to G_s and/or $G_{i/o}$ proteins. In transfected CHO cells with rat β_3 -ARs (Granneman et al., 1991) and murine 3T3-F442A preadipocytes (Fève 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 $G_{i/o}$ 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 $G_{i/o}$ 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 $G_{i/o}$ proteins in rat adult cardiac myocytes (Xiao and Lakatta, 1995).

Then, in order to determine whether $\alpha_{i/o}$ 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 co-injected 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 due to 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 wildtype or microinjected cells untreated with the β_3 -AR agonist. Altogether, 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 previous studies, which reported that β_3 -ARs stimulated the MAPK pathway *via* the activation of $G_{i/o}$ proteins. In CHO/K1 cells overexpressing the human β_3 -AR, the β_3 -AR was simultaneously coupled to G_s and $G_{i/o}$ proteins leading to the activation of adenylyl cyclase and MAPK, respectively (Gerhardt et al., 1999). In HEK293 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). By contrast, in 3T3-L1 adipocytes, Mizuno and colleagues (2000) have proposed that several β_3 -AR agonists (BRL 37344, CGP 12177 and SR 58611) produced a ERK1/2 phosphorylation *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. In our knowledge, this is the first report

relating the regulation of CFTR activity through the MAPK, albeit 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 IL-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.

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 - ERK 1/2 MAPK pathway. Further investigations are clearly necessary in order to determine whether MAPK could modulate CFTR activity in epithelial cells from healthy and CF patients. 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 present 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 where β_1 - and β_2 -ARs are decreased, like in CF (Sharma and Jeffery, 1990).

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Footnotes

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Figure legends

Fig. 1 CFTR regulation by β_3 -AR stimulation in A549 cells. **A-** In cells injected with CFTR (30 $\mu\text{g/ml}$) cDNA, membrane permeability to halides (p in min^{-1}) was measured under baseline (open columns), 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 co-injected with CFTR (30 $\mu\text{g/ml}$) and β_3 -AR (0.1 $\mu\text{g/ml}$) cDNAs, membrane permeability to halides (p in min^{-1}) was measured under baseline and during β_3 -AR stimulation performed by 15 min of perfusion with different concentrations of CGP 12177 (CGP). The functional presence of CFTR was confirmed by application of 10 μM forskolin (Fsk).

Data are the means \pm S.E of 35-86 cells. ***: $P < 0.001$ vs baseline.

Fig. 2 Effect of PKA inhibitors on CFTR regulation by β -AR stimulation. **A-** In A549 cells co-injected with CFTR cDNA (30 $\mu\text{g/ml}$) and β_3 -AR cDNA (0.1 $\mu\text{g/ml}$), membrane permeability to halides (p in min^{-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 $\mu\text{g/ml}$), membrane permeability to halides (p in min^{-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 co-injected with CFTR cDNA (30 $\mu\text{g/ml}$) and β_2 -AR cDNA (0.3 $\mu\text{g/ml}$), membrane permeability to halides (p in min^{-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 \pm S.E. of 25-68 cells. **: $P < 0.01$, ***: $P < 0.001$ vs baseline; ###: $P < 0.001$ vs untreated cells. ns: no significant.

Fig. 3 Effect of PTX pretreatment on CFTR regulation by β_3 -AR stimulation in A549 cells. Cells were injected with CFTR cDNA (30 $\mu\text{g/ml}$) in (A) the absence or (B) the presence of β_3 -AR cDNA (0.1 $\mu\text{g/ml}$). 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 during 36 h).

Data are the means \pm S.E. of 35-39 cells. **: $P < 0.01$, ***: $P < 0.001$ vs baseline.; ###: $P < 0.01$ vs untreated cells. ns: no significant.

Fig. 4 Effect of β -ARK on CFTR regulation by β_3 -AR stimulation in A549 cells. Cells were injected with CFTR (30 $\mu\text{g/ml}$) and β_3 -AR (0.1 $\mu\text{g/ml}$) cDNAs in the absence or the presence of β -ARK cDNA (5 $\mu\text{g/ml}$; an inhibitor of the $\beta\gamma$ complex). Membrane permeability to halides (p in min^{-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 \pm S.E. of 41 cells. ***: $P < 0.001$ vs baseline; ###: $P < 0.01$ vs untreated cells.

Fig. 5 Effect of PI3K inhibitors on CFTR regulation by β_3 -AR stimulation in A549 cells. Cells were injected with CFTR (30 $\mu\text{g/ml}$) and β_3 -AR (0.1 $\mu\text{g/ml}$) 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 (A) 50 μM

wortmannin or **(B)** 10 μ M LY 244002, two PI3K inhibitors. The direct activation of CFTR was obtained by application of 10 μ M forskolin (Fsk).

Data are the means \pm S.E. of 25-33 cells. **: $P < 0.01$, ***: $P < 0.001$ vs baseline, ###: $P < 0.001$ vs 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 μ g/ml) and β_3 -AR (0.1 μ g/ml) cDNAs. Membrane permeability to halides (p in min^{-1}) was measured under baseline and β_3 -AR stimulation performed with 1 μ M CGP 12177 for 15 min (CGP) in cells pretreated or not with **(A)** 20 μ M PD 98059 or **(B)** 10 μ M UO126, two MAPK inhibitors. Direct activation of CFTR was obtained by application of 10 μ M forskolin (Fsk).

Data are the means \pm S.E. of 30-34 cells. **: $P < 0.01$, ***: $P < 0.001$ vs baseline, ###: $P < 0.001$ vs untreated cells.

Fig. 7 Phospho-p44/42 MAPK and β_3 -AR expression in A549 cells in pretreated or not by 1 μ M CGP 12177. Cells were injected with β_3 -AR cDNA (0.1 μ g/ml) and fluorescein isothiocyanate-labeled dextran. Fluorescein allowed to stain successfully microinjected cells nucleus (A and C). β_3 -AR and phospho-p44/42 MAPK antibodies were revealed with peroxydase and alkaline phosphatase conjugated second serums, respectively (B-C). On bright field photomicrographs (X60) performed on cells untreated by CGP 12177, black arrowheads indicate cells labeled with β_3 -AR antibody (brown staining - blue panel; B-C). On cells incubated during 5 min with 1 μ M CGP 12177, blue arrowheads show cells stained in brown with β_3 -AR antibody and in addition strongly labelled in black with Phospho-p44/42 MAPK antibody (red panel; B-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 $\mu\text{g/ml}$) 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 PD 98059, a MAPK inhibitor. The direct activation of CFTR was obtained by application of 10 μM forskolin (Fsk).

Data are the means \pm S.E. of 15-88 cells. ***: $P < 0.001$ vs baseline; #: $P < 0.01$, ###: $P < 0.001$ vs untreated cells.

Fig. 1

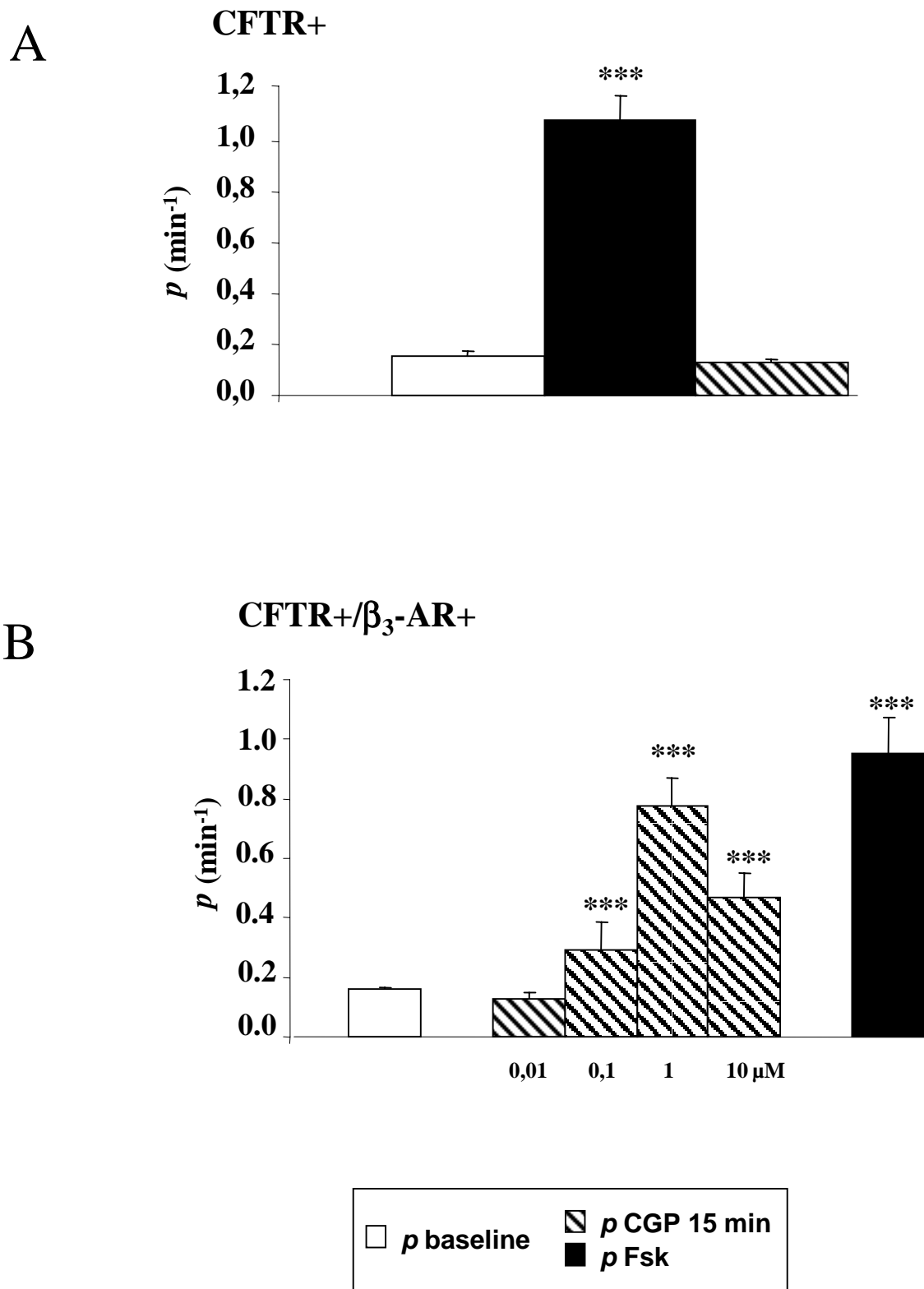
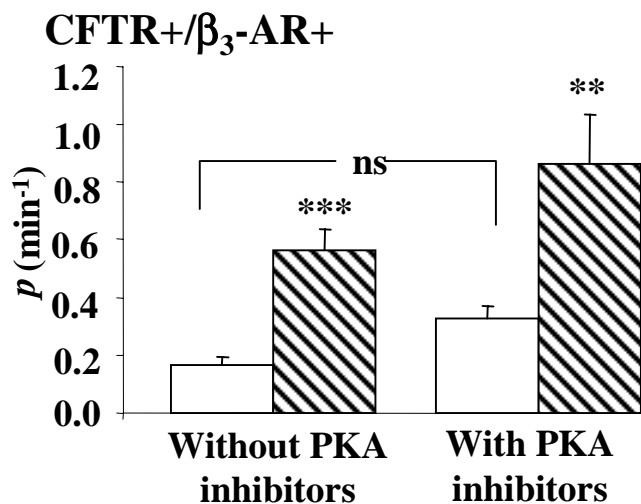
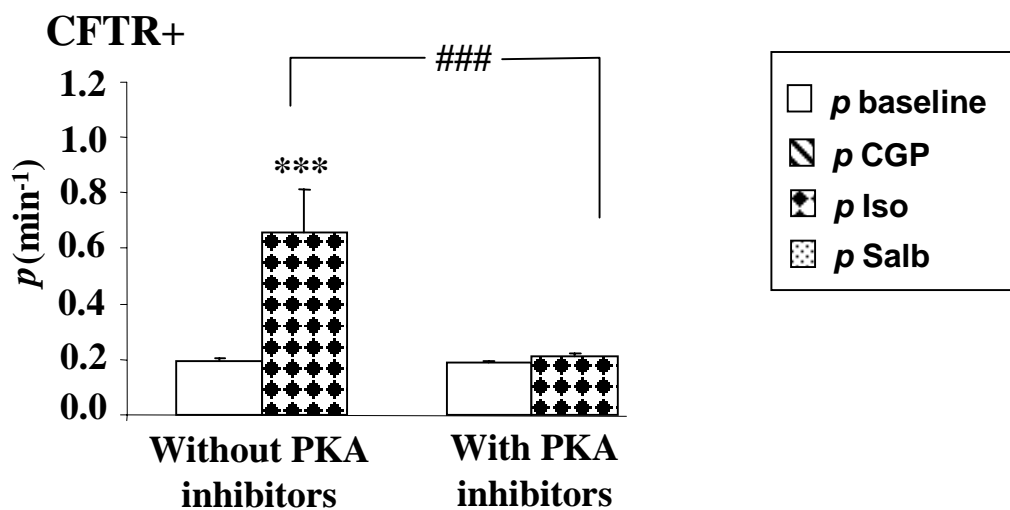


Fig. 2

A



B



C

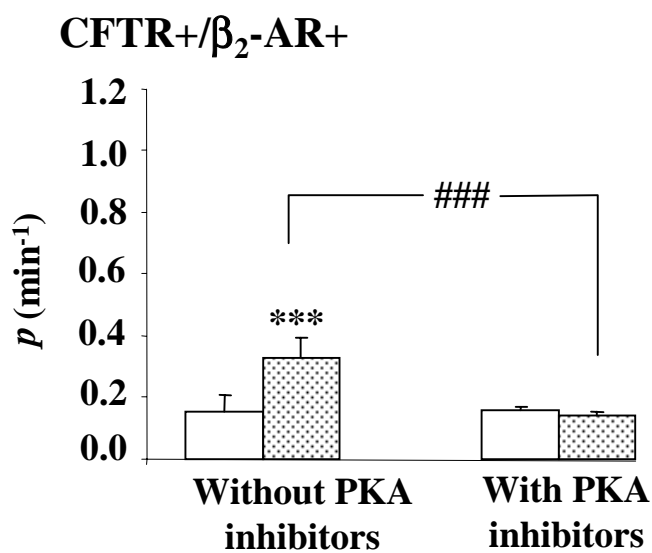
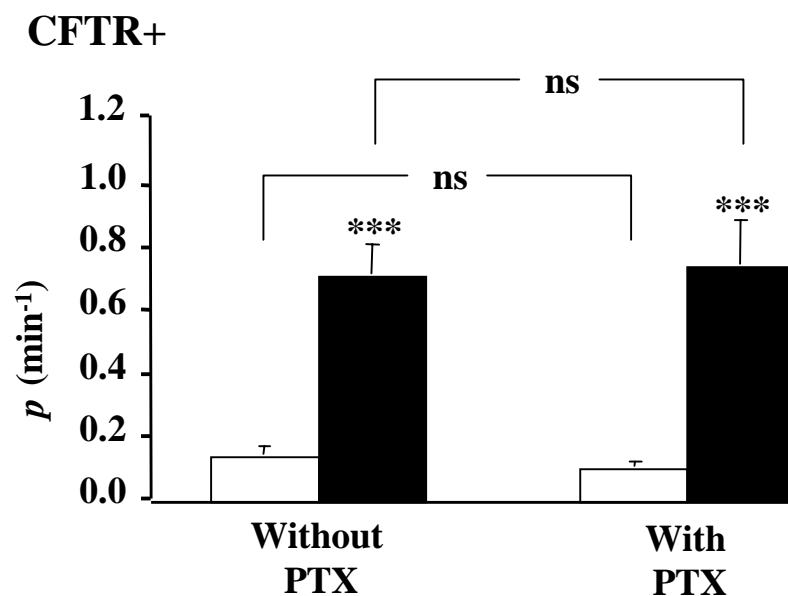


Fig. 3

A



B

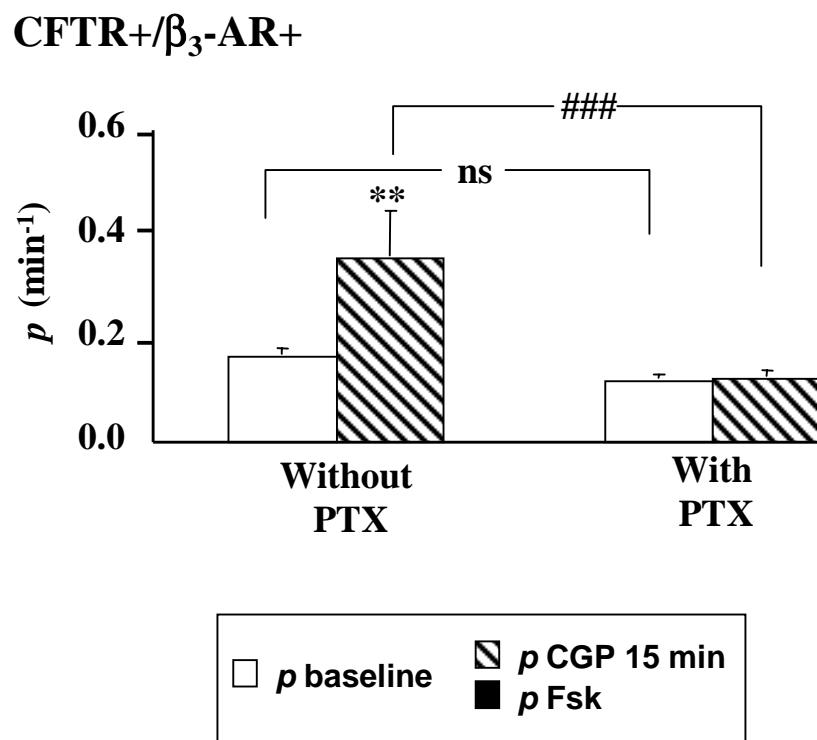


Fig. 4

Inhibitor of $\beta\gamma$ subunits (5 $\mu\text{g/ml}$)

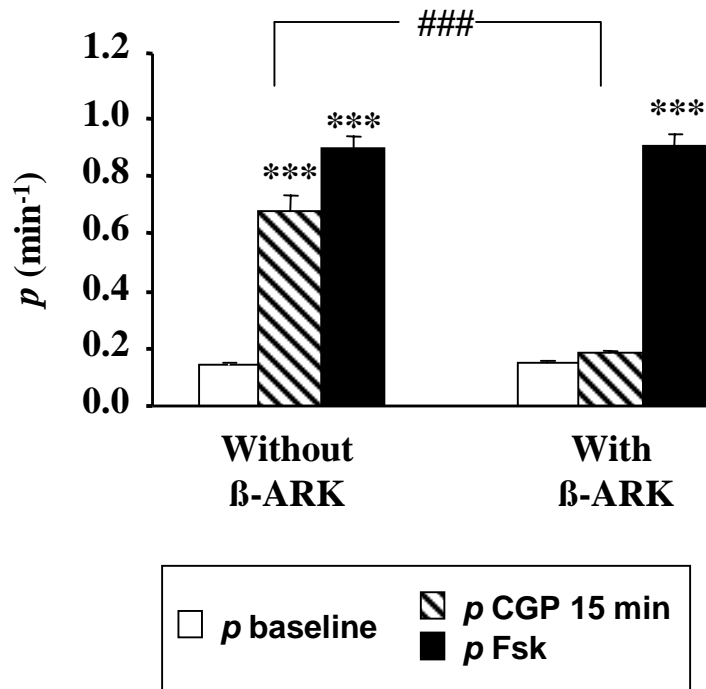


Fig. 5

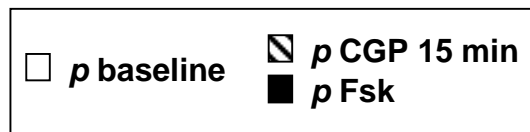
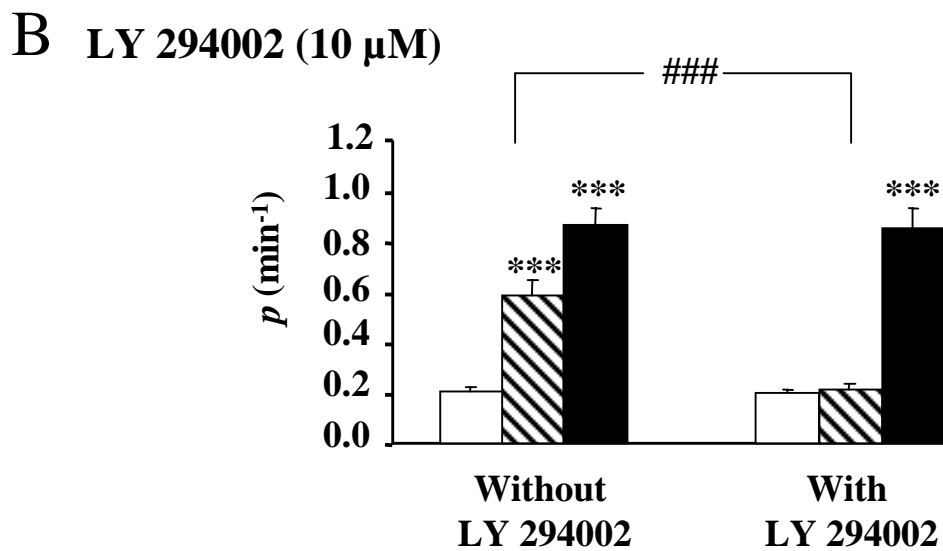
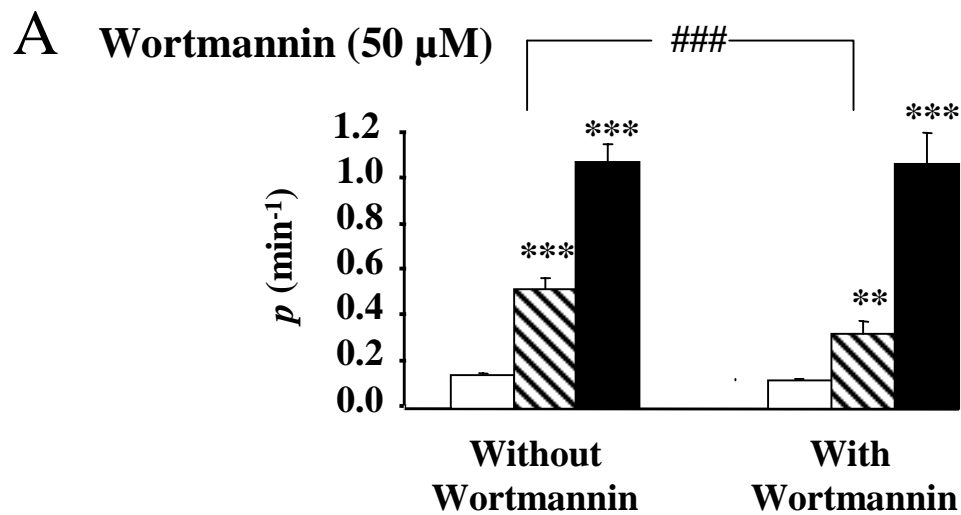


Fig. 6

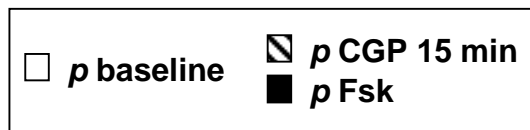
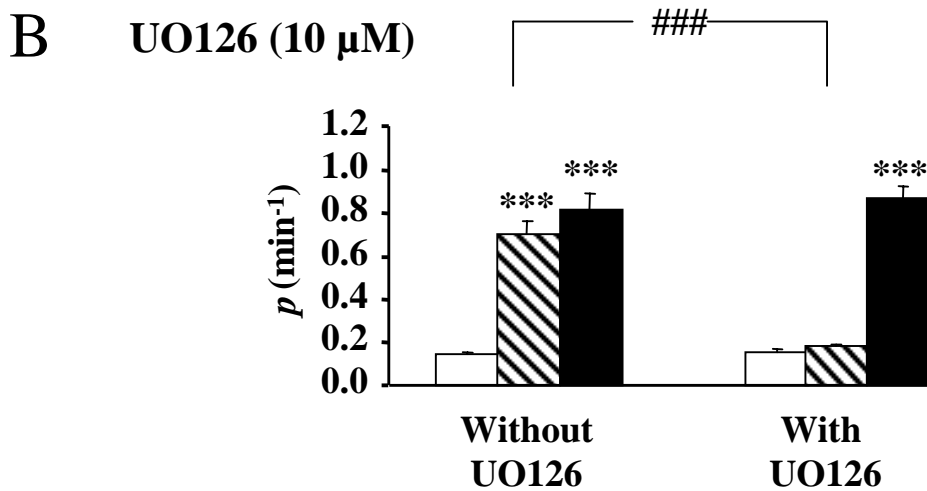
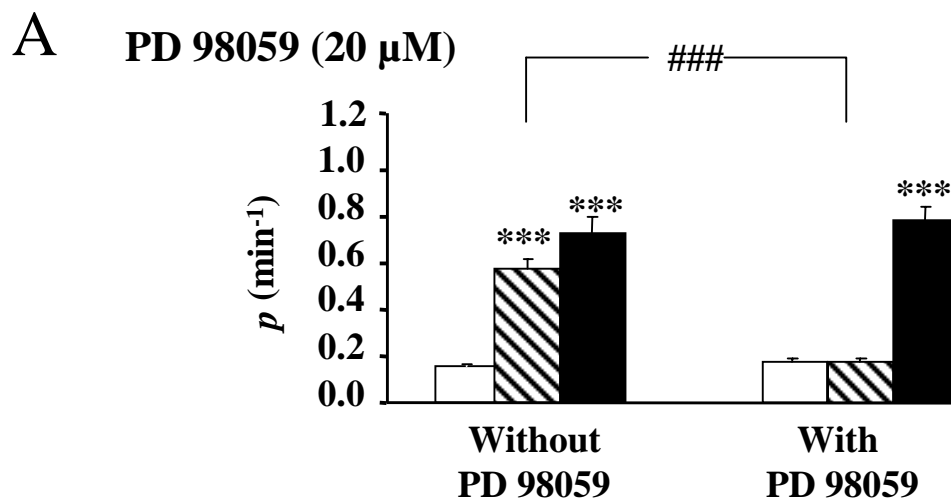


Fig. 7

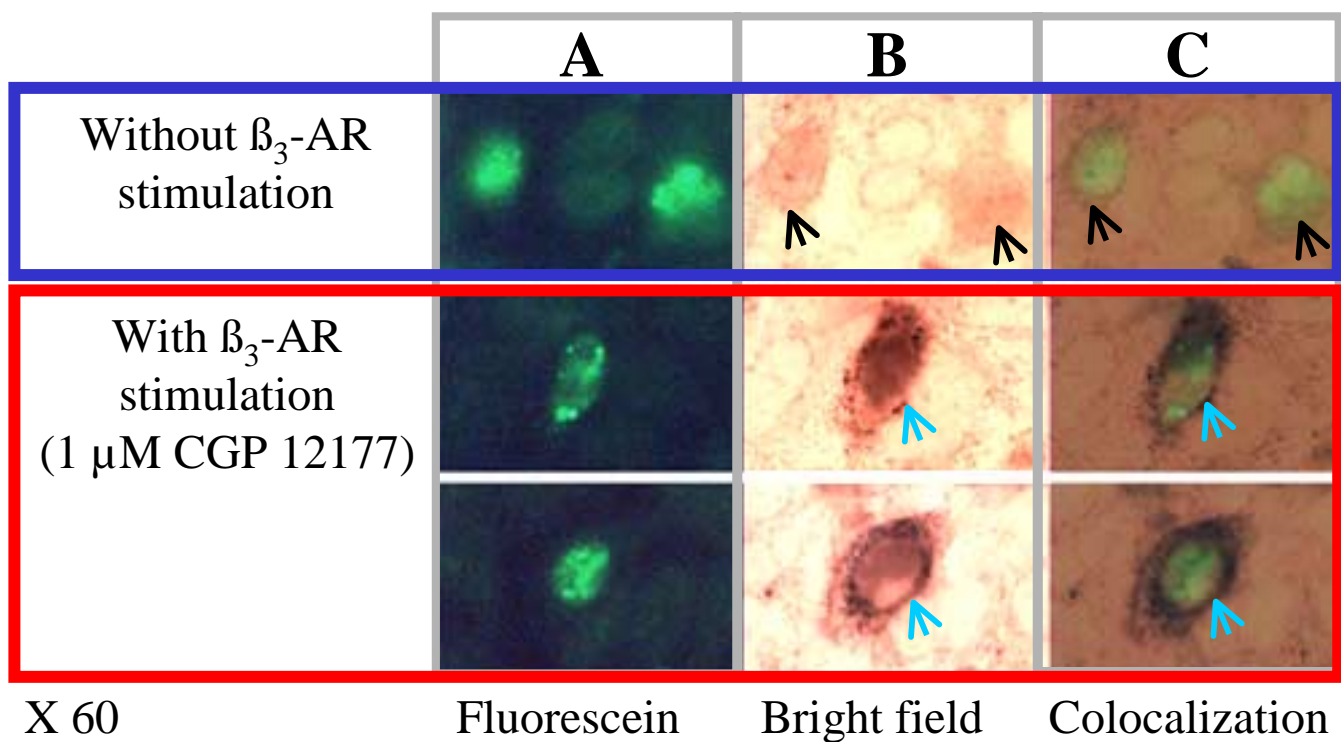
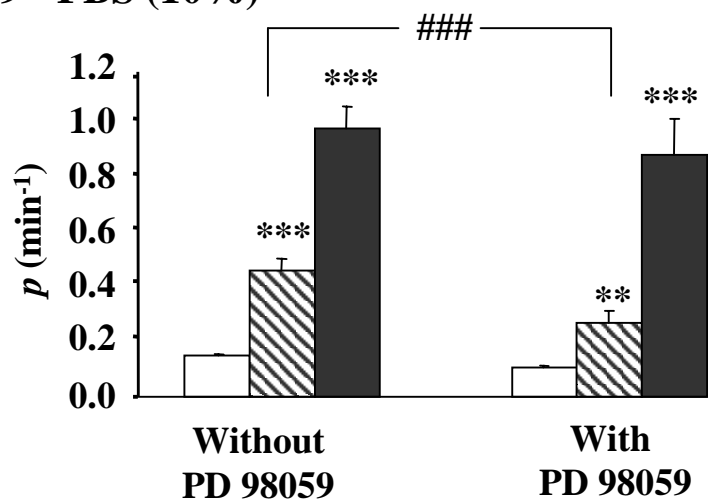


Fig. 8

A A549 - FBS (10%)



B T84 - FBS (10%)

