Molecular Pharmacology Fast Forward. Published on November 24, 2004 as doi:10.1124/mol.104.002097


MOLPHARM/2004/002097

# 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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Molecular Pharmacology Fast Forward. Published on November 24, 2004 as DOI: 10.1124/mol.104.002097
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**Running Title**: Regulation of CFTR by MAPK pathway.

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Manuscript information:

Number of text pages: 28

Number of Figures: 8

Number of Tables: 0

Number of words in the abstract: 250

Number of words in the introduction: 474

Number of words in Discussion: 1118

Number of Reference: 35

**Abbreviations:** A549, human lung epithelial-derived cell line; ß-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, fœtal bovine serum; FITC, Fluorescein isothiocyanate; FSK, forkolin; 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.

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## Abstract

We have previously shown that in a heterologous mammalian expression system, A549 cells, $\beta_3$-adrenoceptor ($\beta_3$-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 $\beta_3$-AR. CFTR activity was functionally assessed by microcytofluorimetry. The application of 1 µM CGP 12177, a ß$_3$-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 βγ subunits, abolished the CGP 12177-induced CFTR activation, suggesting the involvement of βγ 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 $\beta_3$-AR exhibited MAPK activation in response to CGP 12177. Furthermore, CFTR activity increased in cells pretreated with 10% fœtal 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 $\beta_3$-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 $\beta_3$-ARs. This pathway represents a new regulation for CFTR.

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## 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 (ß-AR) stimulation is well known to regulate the intracellular cAMP levels. ß-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 $ß_2$-AR stimulation in those cells. However, in *xenopus* oocytes, the $ß_2$-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 ß$_3$-AR stimulation produced either by isoproterenol (a non selective ß-AR agonist) in the presence of nadolol (a ß$_1$, ß$_2$-AR antagonist), or SR 58611A (a preferential ß$_3$-AR agonist), or CGP 12177 (a partial ß$_3$-AR agonist) (Leblais et al., 1999). This CFTR activation induced by ß$_3$-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 ß$_3$-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).

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## 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 an humified 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, the $pcDNA_3$-ß$_2$ and $pcDNA_3$-ß$_3$A plasmids (gifts from D. Langin, Toulouse, France) encoded human ß$_2$-AR and A isoform of the human ß$_3$-AR, respectively. The $pcDNA_3$-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 ß$_3$-AR and or 0.3 µg/ml for human ß$_2$-AR ) at 1 day after plating on glass coverslips (Nunclon; 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.

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**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 $Ca^{2+}$-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 (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 $MgCl_2$, 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 mediums. Iodure ($I^-$) and nitrate ($NO_3^-$) containing mediums were identical to the extracellular solution except that $I^-$ and $NO_3^-$ 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-Fo) / Fo × 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, was used as an index of CFTR activity.

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**Immunohistochemical assays.** After injection, cells were placed for 12 h in a medium containing 10% fœtal 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 µM 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 ß$_3$-AR (a gift from GlaxoBeecham, Harlow, UK) for 3 h at room temperature. This antiserum was revealed with peroxydase conjugated second serum. After the control of the efficiency of the ß$_3$-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 µM forskolin plus 100 µM 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%.

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**Statistical analysis.** Data are expressed as the means ± 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.

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## Results

**CFTR activation by ß$_3$-AR stimulation.** In A549 cells injected only with the plasmid encoding human CFTR, the application of 10 µM forkolin, a direct activator of adenylate cyclase, increased the *p* value by 8-fold whereas the application of 1 µM CGP 12177 did not modified basal CFTR activity (Fig.1A). In A549 cells co-expressing CFTR and ß$_3$-AR, the CFTR basal permeability to halides (*p*) was 0.15±0.01 min$^{-1}$. The application of increasing concentrations (0.01 to 10 µM) of CGP 12177 (a ß$_3$-AR agonist) produced an increase of *p* values in concentration-dependent manner. The maximal effect was obtained at 1 µM. 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 µM.

**Involvement of G$_{i/o}$ protein in CFTR activation induced by ß$_3$-AR stimulation.** To determine the putative involvement of PKA in the CFTR regulation by ß$_3$-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 non-selective ß-AR agonist, and salbutamol, a ß$_2$-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 ß$_1$- and/or ß$_2$-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 ß$_2$-AR, salbutamol at 10 µM 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-

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expressing CFTR and ß$_3$-AR, the response to CGP 12177 was fully abolished after pretreatment by PTX (Fig.3B), suggesting that CFTR activation by ß$_3$-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 ß-ARK (5 µg/ml), an inhibitor of the ßγ complex (Koch et al., 1994), in addition to plasmids encoding for CFTR and ß$_3$-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 ß$_3$-AR stimulation**. In cells pretreated with 50 µM 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 µM 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 µM PD 98059 (Fig.6A) or 10 µM 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 ß$_3$-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 µM CGP 12177 during 5 min. In a first step, an incubation with the antibody raised against human ß$_3$-AR was performed. We verified that the non-injected cells did not present any staining whereas cells injected with ß$_3$-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 ß$_3$-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 ß$_3$-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).

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## Discussion

We have previously demonstrated that ß$_3$-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 ß$_3$-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 ß$_3$-AR stimulation.

ß$_3$-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 ß$_3$-AR agonist, CGP 12177, activated only ß$_3$-ARs in our recombinant system. This increase of the CFTR activity induced by ß$_3$-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 ß$_1$- and ß$_2$-ARs by isoproterenol was abolished in PKA inhibitor-pretreated cells. Furthermore, in cells co-expressing CFTR and ß$_2$-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 ß$_2$-AR did not disturb its coupling to cAMP/PKA pathway. Thus, our results demonstrated that ß$_3$-AR did not link to the cAMP/PKA pathway in A549 cells although this pathway was present and functional.

CFTR activation by the ß$_3$-AR stimulation was abolished by the pretreatment with PTX, indicating that PTX sensitive-G proteins, $G_{i/0}$, were involved in this effect. Several studies

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performed in different cellular types or tissues have already reported that ß$_3$-ARs could be linked to $G_s$ and/or $G_{i/0}$ proteins. In transfected CHO cells with rat ß$_3$-ARs (Granneman et al., 1991) and murine 3T3-F442A preadipocytes (Fève et al., 1991), ß$_3$-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 $G_s$, ß$_3$-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, ß$_3$-ARs were shown to be exclusively linked to $G_{i/0}$ proteins (Gauthier et al., 1996). The coupling of ß$_3$-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 ß$_2$-AR has been shown to activate both $G_s$ and $G_{i/0}$ proteins in rat adult cardiac myocytes (Xiao and Lakatta, 1995).

Then, in order to determine whether $\alpha_{i/0}$ subunit and/or ßγ complex of the $G_{i/o}$ protein were involved in CFTR activation induced by the ß$_3$-AR stimulation, A549 cells were co-injected with the plasmid encoding the C-terminal region of ß-ARK, an inhibitor of the ßγ complex, in addition to the plasmids encoding CFTR and ß$_3$-ARs. In such conditions, the CFTR activation induced by the ß$_3$-AR stimulation was fully abolished indicating a total regulation of CFTR by the ßγ complex in our model. It should be noted that several studies reported that CFTR activity could be regulated by the $\alpha_i$ subunit. In cultured human airway epithelial cells, the purified $\alpha_i$ 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 ßγ complex, the inhibition of CFTR-induced current was abolished, probably due to the assembly of the different subunits in an inactive αßγcomplex (Stow et al., 1991).

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In the present study, the pretreatment of A549 cells by selective inhibitors of PI3K and ERK1/2 MAPK abolished the CFTR activation induced by ß$_3$-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 ß$_3$-AR agonist. This labeling was absent in wildtype or microinjected cells untreated with the ß$_3$-AR agonist. Altogether, these results demonstrate that ß$_3$-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 ß$_3$-ARs stimulated the MAPK pathway *via* the activation of $G_{i/0}$ proteins. In CHO/K1 cells overexpressing the human ß$_3$-AR, the ß$_3$-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 HEK293 cells expressing human ß$_3$-AR as well as in intact 3T3-F442A adipocytes, CL 316243, a ß$_3$-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 ß$_3$-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 ß$_3$-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 ß$_3$-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 $\beta_3$-AR, exists. These findings might play an important role in airway pathologies where $ß_1$- and $ß_2$-ARs are decreased, like in CF (Sharma and Jeffery, 1990).

## Acknowledgments

We thank Dr Jacques Noireaud for reading and criticizing the manuscript and Karine Laurent and Mortéza Erfanian for their expert technical assistance with cell cultures.

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## Footnotes

[$] This work was supported by grants from the Association "Vaincre la Mucoviscidose" and the “Fondation Langlois”.

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## Figure legends

**Fig. 1 CFTR regulation by $\beta_3$-AR stimulation in A549 cells**. **A-** In cells injected with CFTR (30 µg/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 ß$_3$-AR agonist. **B-** In cells co-injected with CFTR (30 µg/ml) and $\beta_3$-AR (0.1 µg/ml) cDNAs, membrane permeability to halides (*p* in $min^{-1}$) was measured under baseline and during $\beta_3$-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 ± 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 µg/ml) and $\beta_3$-AR cDNA (0.1 µg/ml), membrane permeability to halides (*p* in $min^{-1}$) was measured under $\beta_3$-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 $min^{-1}$) 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 µg/ml) and $\beta_2$-AR cDNA (0.3 µg/ml), membrane permeability to halides (*p* in $min^{-1}$) was measured under $\beta_2$-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 25-68 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 $\beta_3$-AR stimulation in A549 cells**. Cells were injected with CFTR cDNA (30 µg/ml) in **(A)** the absence or **(B)** the presence of $\beta_3$-AR cDNA (0.1 µg/ml). *p* value was measured under baseline and under application of 10 µM forskolin (Fsk) or during $\beta_3$-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 ± 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 $\beta_3$-AR stimulation in A549 cells**. Cells were injected with CFTR (30 µg/ml) and $\beta_3$-AR (0.1 µg/ml) cDNAs in the absence or the presence of β-ARK cDNA (5 µg/ml; an inhibitor of the βγ complex). Membrane permeability to halides (*p* in $min^{-1}$) was measured during baseline and after $\beta_3$-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 *vs* baseline; $^{\#\#\#}$: P<0.01 *vs* untreated cells.

**Fig. 5 Effect of PI3K inhibitors on CFTR regulation by $\beta_3$-AR stimulation in A549 cells**. Cells were injected with CFTR (30 µg/ml) and $\beta_3$-AR (0.1 µg/ml) cDNAs. Membrane permeability to halides (*p* in $min^{-1}$) was measured under baseline and $\beta_3$-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 µM LY 244002, two PI3K inhibitors. The direct activation of CFTR was obtained by application of 10 µM forskolin (Fsk).

Data are the means ± S.E. of 25-33 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 $\beta_3$-AR stimulation in A549 cells.** Cells were injected with CFTR (30 µg/ml) and $\beta_3$-AR (0.1 µg/ml) cDNAs. Membrane permeability to halides (*p* in $min^{-1}$) was measured under baseline and $\beta_3$-AR stimulation performed with 1 µM CGP 12177 for 15 min (CGP) in cells pretreated or not with **(A)** 20 µM PD 98059 or **(B)** 10 µM UO126, two MAPK inhibitors. Direct activation of CFTR was obtained by application of 10 µM forskolin (Fsk).

Data are the means ± 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 ß$_3$-AR expression in A549 cells in pretreated or not by 1 µM CGP 12177.** Cells were injected with $\beta_3$-AR cDNA (0.1 µg/ml) and fluorescein isothiocyanate-labeled dextran. Fluorescein allowed to stain successfully microinjected cells nucleus (A and C). ß$_3$-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 ß$_3$-AR antibody (brown staining - blue panel; B-C). On cells incubated during 5 min with 1 µM CGP 12177, blue arrowheads show cells stained in brown with ß$_3$-AR antibody and in addition strongly labelled in black with Phospho-p44/42 MAPK antibody (red panel; B-C).

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**Fig. 8 Activation of CFTR by the MAPK pathway independently of $\beta_3$-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 PD 98059, a MAPK inhibitor. The direct activation of CFTR was obtained by application of 10 µM forskolin (Fsk).

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

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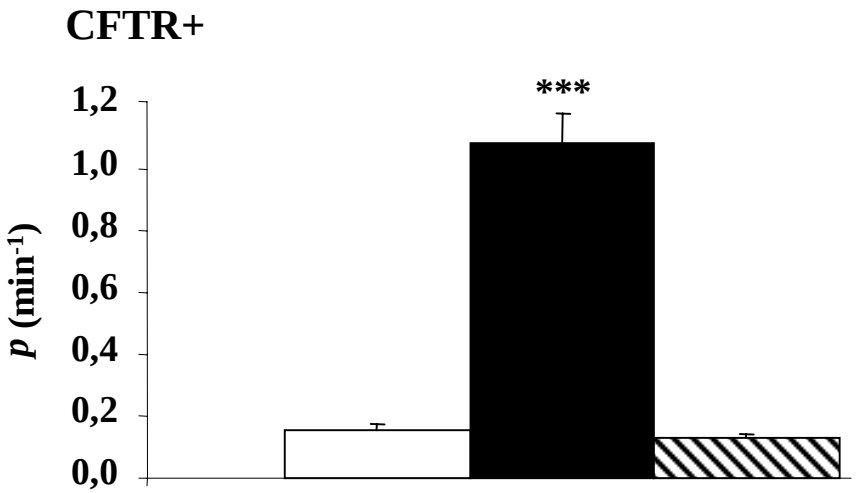
Fig. 1

A




B

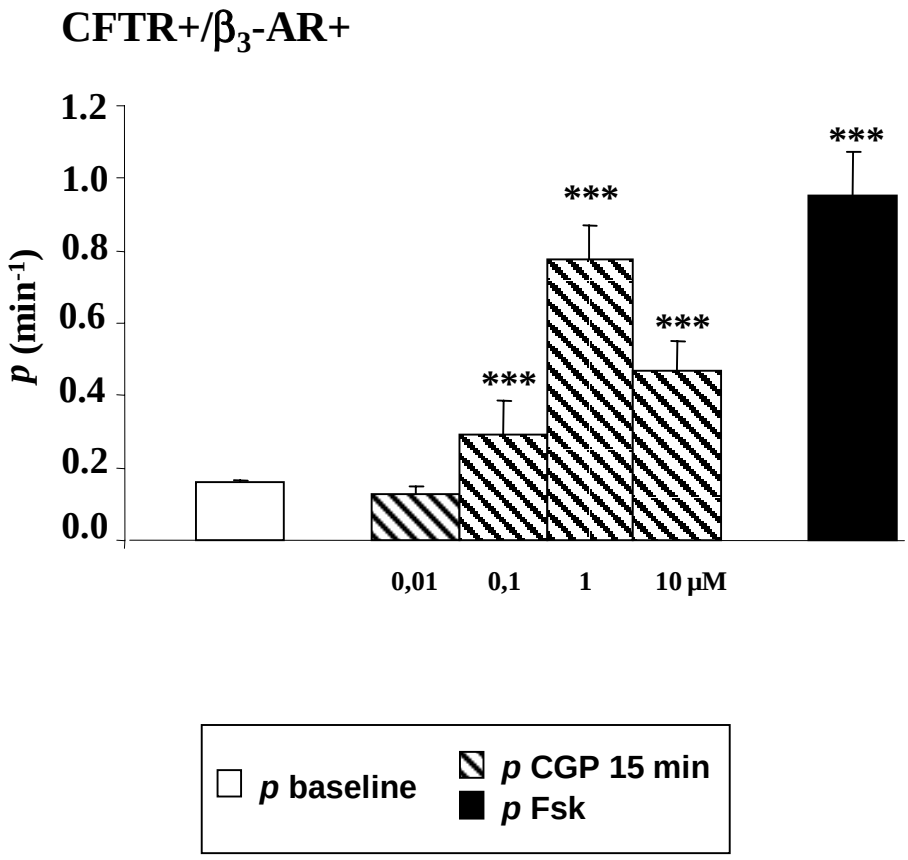


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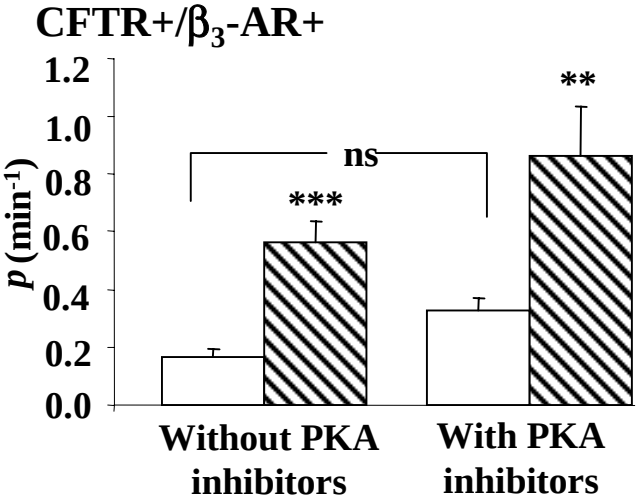
Fig. 2

A

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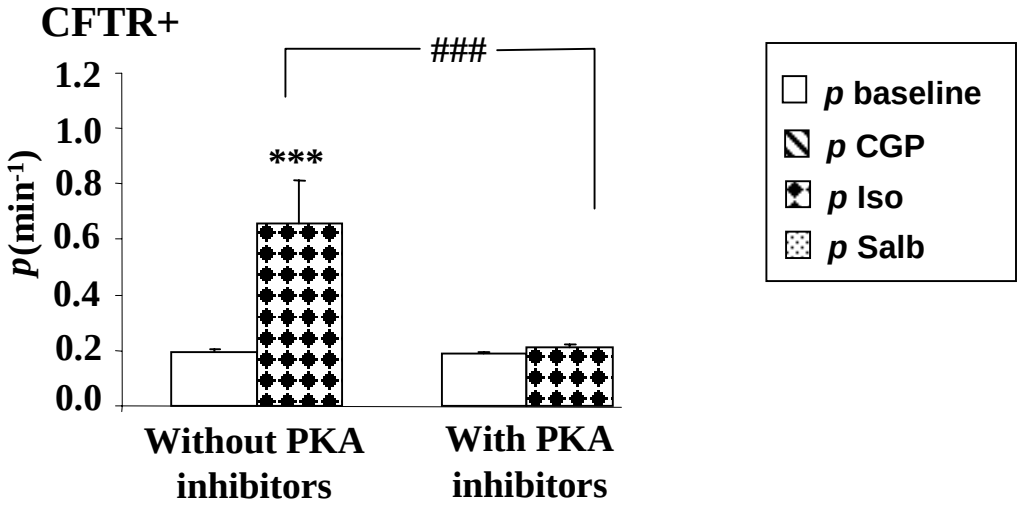


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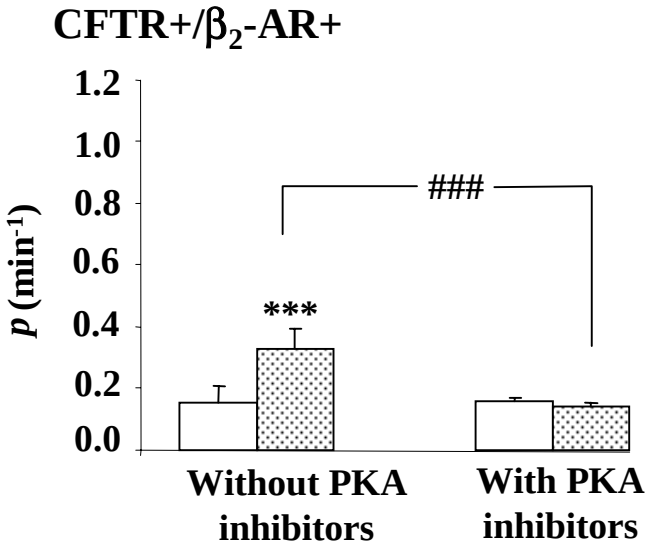

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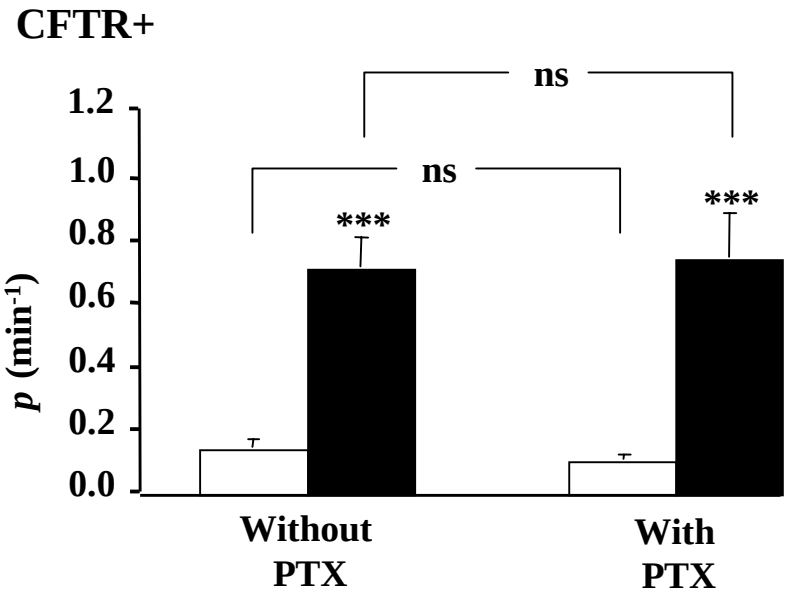
Fig. 3

A

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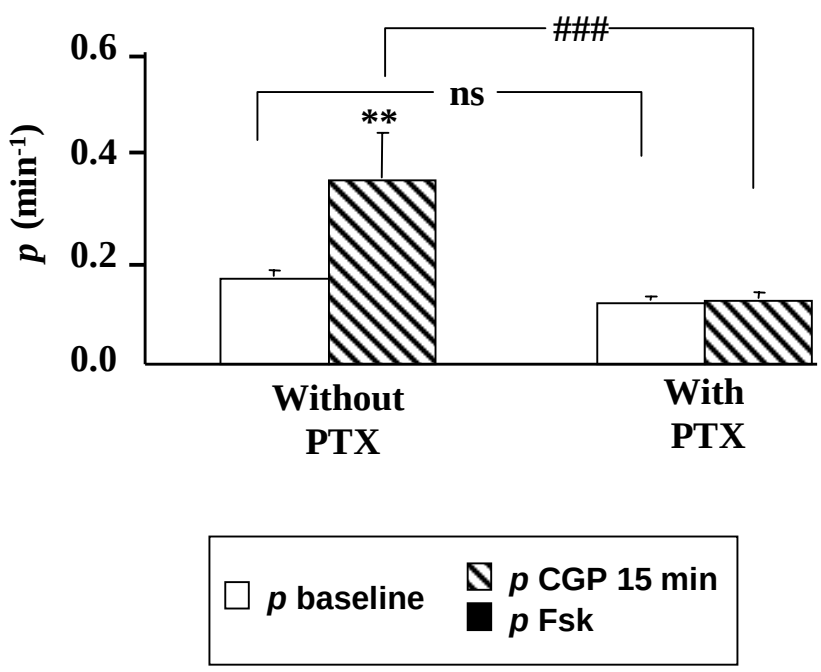

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Fig. 4

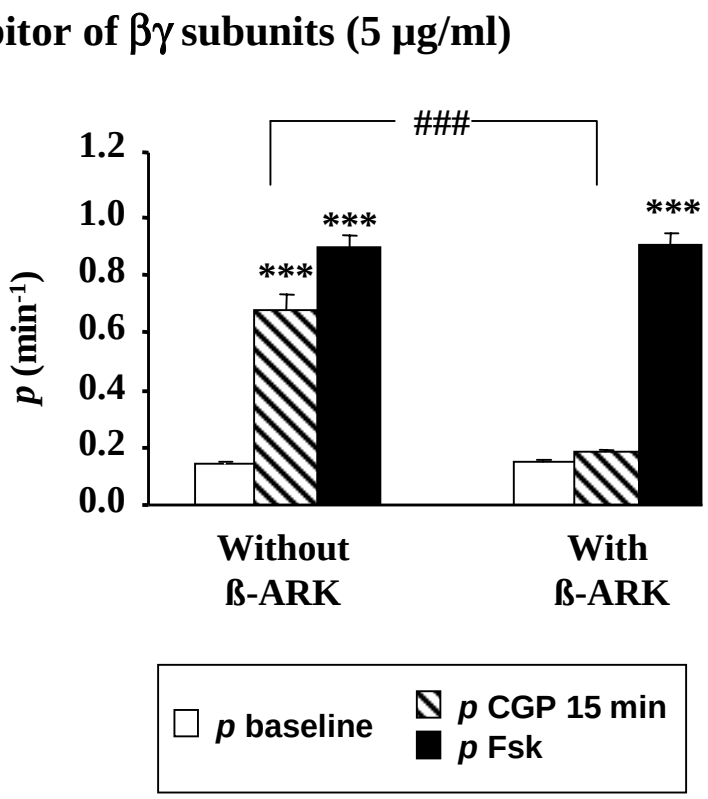

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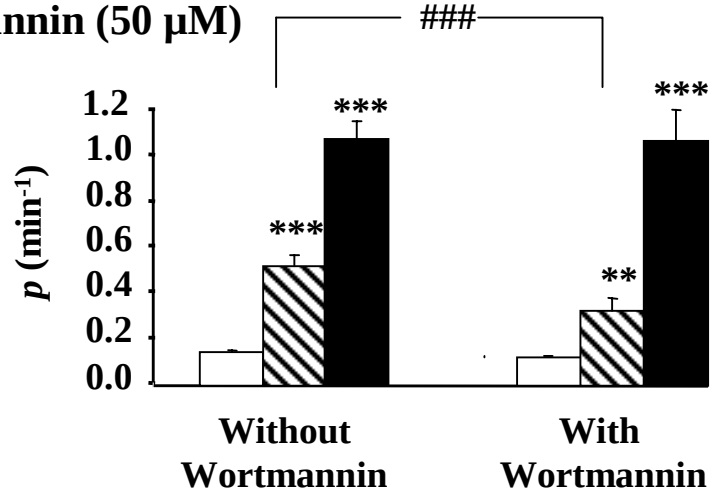
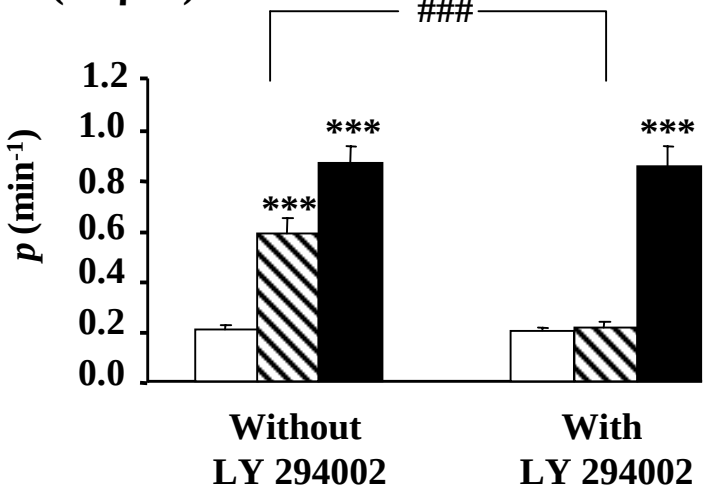
Fig. 5

A **Wortmannin (50 µM)**




B **LY 294002 (10 µM)**



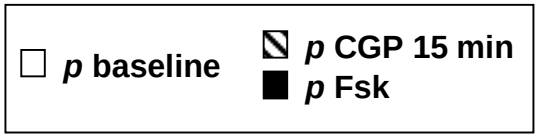



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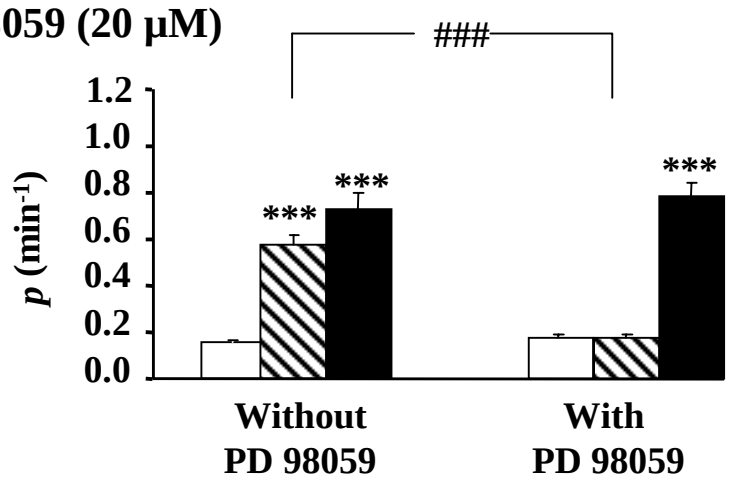
Fig. 6

A

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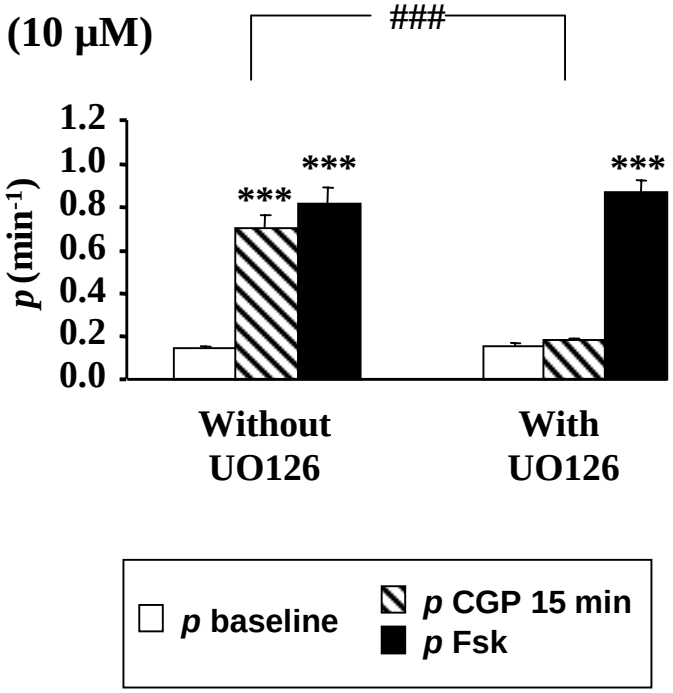

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Fig. 7

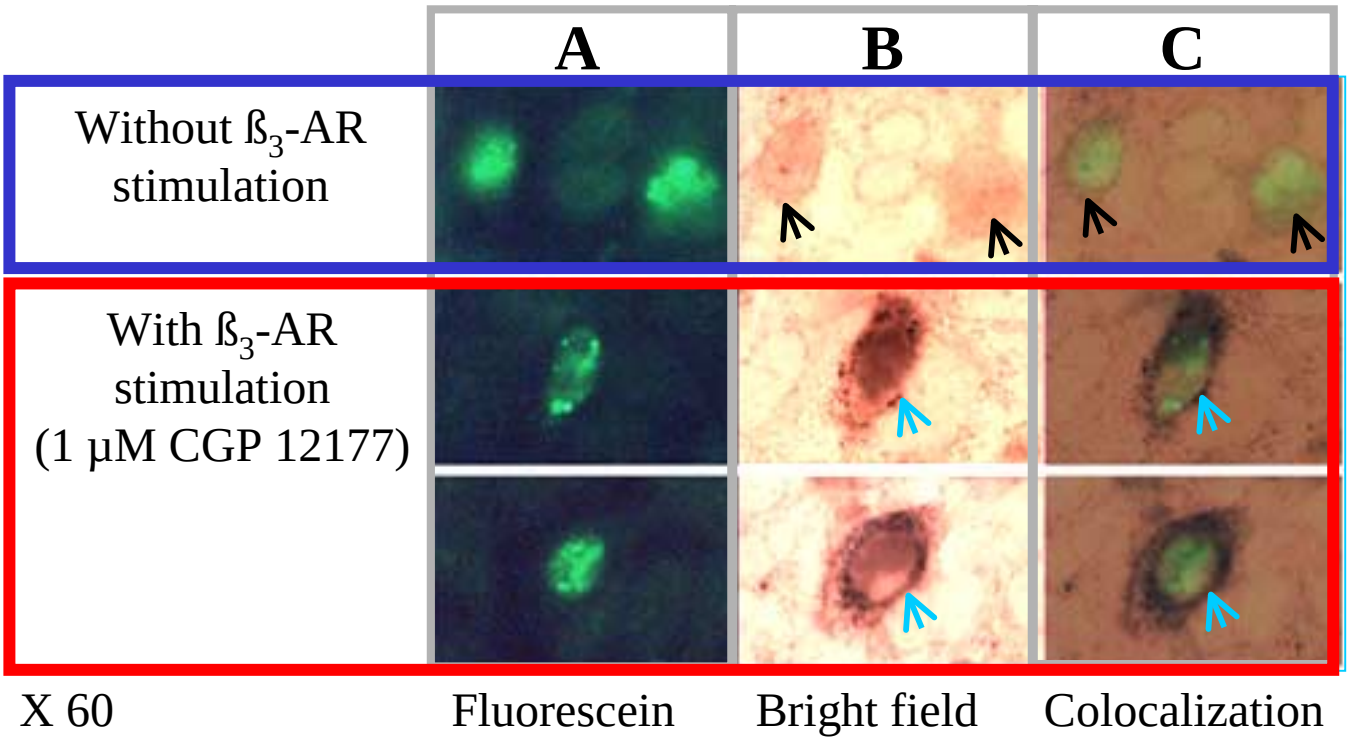

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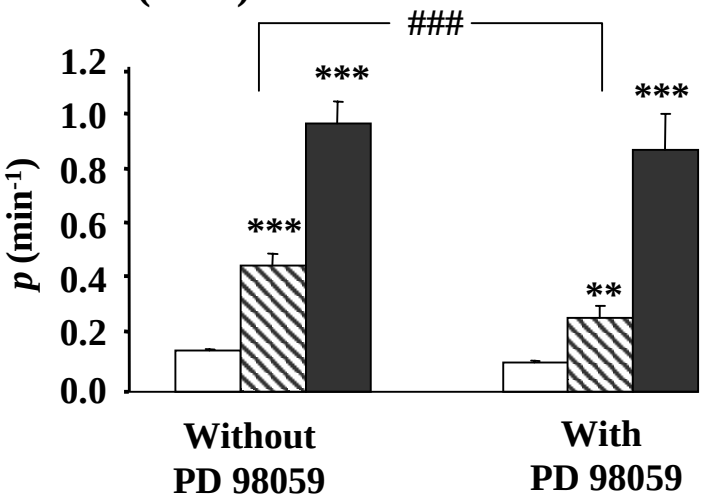
Fig. 8

A **A549 - FBS (10%)**




B **T84 - FBS (10%)**