β3-Adrenoceptor Control the Cystic Fibrosis Transmembrane Conductance Regulator through a cAMP/Protein Kinase A-independent Pathway*

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In human cardiac myocytes, we have previously identified a functional β3-adrenoceptor in which stimulation reduces action potential duration. Surprisingly, in cardiac biopsies obtained from cystic fibrosis patients, β3-adrenoceptor agonists produced no effects on action potential duration. This result suggests the involvement of cystic fibrosis transmembrane conductance regulator (CFTR) chloride current in the electrophysiological effects of β3-adrenoceptor stimulation in non-cystic fibrosis tissues. We therefore investigated the control of CFTR activity by human β3-adrenoceptors in a recombinant system: A549 human cells were intranuclearly injected with plasmids encoding CFTR and β3-adrenoceptors. CFTR activity was functionally assayed using the 6-methoxy-N-(3-sulfopropyl)quinolinium fluorescence probe and the patch-clamp technique. Injection of CFTR-cDNA alone led to the expression of a functional CFTR protein activated by cAMP or cGMP. Co-expression of CFTR (but not of mutated ΔF508-CFTR) with high levels of β3-adrenoceptor produced an increased halide permeability under base-line conditions that was not further sensitive to cAMP or β3-adrenoceptor stimulation. Patch-clamp experiments confirmed that CFTR channels were permanently activated in cells co-expressing CFTR and a high level of β3-adrenoceptor. Permanent CFTR activation was not associated with elevated intracellular cAMP or cGMP levels. When the expression level of β3-adrenoceptor was lowered, CFTR was not activated under base-line conditions but became sensitive to β3-adrenoceptor stimulation (isoproterenol plus nadolol, SR 58611, or CGP 12177). This later effect was not prevented by protein kinase A inhibitors. Our results provide molecular evidence that CFTR but not mutated ΔF508-CFTR is regulated by β3-adrenoceptors expression through a protein kinase A-independent pathway.

β3-Adrenoceptors differ from β1- and β2-adrenoceptor subtypes by their molecular structure and pharmacological profile (for review see Ref. 1). The β3-adrenoceptor gene contains two introns (2, 3) leading to alternative splice isoforms, whereas β1- and β2-adrenoceptor genes are intronless. β3-Adrenoceptors are G protein-coupled receptors that interact with either G1 or G2 proteins (4, 5). Depending on the tissue, β3-adrenoceptor stimulation produces functional effects that are either comparable with or opposite to those produced by β1- and β2-adrenoceptor stimulation. For instance, in adipose tissue, β3-adrenoceptor stimulation increases lipolysis through an elevation in intracellular cAMP concentration (6, 7) as does β1- or β2-adrenoceptor stimulation. In the human heart, we have previously demonstrated that β3-adrenoceptors mediate negative inotropic effects (5) in stark contrast to the classical positive inotropic effects caused by β1- and β2-adrenoceptor stimulation. Negative inotropy as produced by β3-adrenoceptor stimulation is unlikely to be related to stimulation of the cAMP pathway but rather to stimulation of the cGMP pathway (8) and is associated with an acceleration in the relaxation phase of the twitch and with a shortening of the action potential duration (5).

The present study issued from the observation that in myocardial samples from cystic fibrosis patients, β3-adrenoceptor stimulation produced negative inotropic effects but remarkably did not shorten the action potential. Cystic fibrosis is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene encoding an ATP-binding cassette protein (9) with anionic channel properties (10) and expressed in the human heart (11). Therefore, the simplest explanation accounting for our observation was that β3-adrenoceptors in non-cystic fibrosis tissues control a repolarizing CFTR chloride conductance lacking in cystic fibrosis tissues. We confirmed this hypothesis using various techniques in a heterologous mammalian expression system.

**EXPERIMENTAL PROCEDURES

Action Potential Recordings—Human ventricular biopsies were obtained from three cystic fibrosis (CF) patients homozygous for the ΔF508 mutation who underwent cardiopulmonary transplantation and from five non-CF patients used as control. Heart and tissues were placed in a transport solution and conveyed rapidly to the laboratory. Preparations were dissected and placed in an experimental chamber. They were superfused at a flow rate of 5 ml/min with oxygenated (95%
O₂, 5% CO₂) Tyrode solution (37 ± 0.5 °C) composed as follows: 120 mM NaCl, 5 mM KCl, 2.7 mM CaCl₂, 1.1 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM glucose, and 20 mM NaHCO₃. Tissues were equilibrated for 60 min and then subjected to field stimulation at a pacing cycle length of 1,700 ms. Action potentials were recorded as described previously (12) using concentric needle electrodes, the signal amplified and filtered (300 Hz to 3 kHz), and the action potential and the rate of spontaneous firing were measured.

Cell Cultures—The human lung epithelial-derived cell line A549, the Afadase green monkey kidney-derived cell line COS-7, and the human colon adenocarcinoma cell line T84 were obtained from the American Type Culture Collection (Manassas, VA). A549 and COS-7 cells were cultured in 95% air, 5% CO₂ at 37 °C and passaged weekly.

Plasmids—Transgene DNA plasmids were subcloned into pCEC or pcDNA mammalian expression vectors under the control of a SV40 enhancer/promoter or a cytomegalovirus promoter, respectively. pCEC-CFTR (a kind gift from P. Fahren, INSERM, Créteil, France) and pcDNA-CFTR (a gift from J. P. Behr, CNRS, France) plasmids encoded the wild-type CFTR protein. pcDNA-AF508-CFTR plasmid (also a gift from J. Ricardo) encoded the mutated AF508-CFTR protein. Alternative splicing of the β₁-adrenoceptor mRNA could generate three isoforms with different C-terminal regions: (i) the A isoform is the shortest protein; (ii) the B isoform, which corresponds to the A isoform extended by 12 amino acids in the C-terminal region, is the only isoform expressed in rat and is supposedly not expressed in human; and (iii) the C isoform corresponds to the A isoform extended by 6 amino acids in the C-terminal region and is the prevalent isoform in humans (2, 14). As the C-terminal region of the β₁-adrenoceptor is involved in the coupling with G proteins (1), plasmids encoding either for the A (pcDNA-β₁A) and C (pcDNA-β₁C) isoforms present in human tissues were generated and expressed in mammalian cells. For control experiments, we used a CEEC plasmid lacking the insert and a pcDNA-HOM, plasmid encoding a renal outer medulla potassium channel (a gift from S. C. Hebert, Vanderbilt University, Nashville, TN). For microtuborumetry and patch-clamp experiments, cells settled on glass coverslips (Nunclon; InterMed Nunc, Roskilde, Denmark) were microinjected with plasmids at day 1 or 2 after plating. Our protocol to intracellularly microinject primary smooth muscle cells using the ECET micromanipulator 5246 system and the ECET microinjector 5171 system has been reported in detail elsewhere (13). Plasmids were diluted at various final concentrations (0.1–100 μg/ml) in an injection buffer made of 50 mM NaOH, 50 mM Hepes, pH 7.4, with NaOH, supplemented with 0.5% fluorescein isothiocyanate-dextran to visualize successfully injected cells. The method we used for cell transfection with the 22-kDa polyethylenimine synthetic vector (a gift from S. C. Hebert, Vanderbilt University, Nashville, TN) was previously described (2). Cells were microinjected with DNA plasmids at day 1 or 2 after plating. The Cl⁻ channel activity of CFTR was assessed using the halide-sensitive fluorescent probe SPQ (Molecular Probes, Eugene, OR) as described previously (12). 24 h post-injection or transfection, cells placed on glass coverslips were loaded with SPQ using a microinjection system. The coverslip was mounted on the stage of an inverted microscope (Diaphot; Nikon, 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 (Hamamatsu; ISIS camera system; Photonic Science, Bishopsbridge, United Kingdom) connected to a digital image processing board, the signal amplified and filtered (300 Hz to 3 kHz), and the action potential and the rate of spontaneous firing were measured.

RESULTS
β₁-Adrenoceptor Stimulation Does Not Shorten Action Potential in CF Cardiac Myocytes—In control human endomyocardial tissues from non-CF patients, the specific β₁-adrenoceptor agonist BRL 37344 (3 μM) reduced the action potential duration by −13.8 ± 3.8% (n = 5; p < 0.01) and also slightly reduced the action potential amplitude (−3.7 ± 0.4%; p < 0.05). These effects were not observed in eight myocardial preparations obtained from AF508/Δ508 CF patients, suggesting that the electrophysiological effects of β₁-adrenoceptor stimulation in non-CF tissues were mediated by activation of a chloride repolarizing current flowing through CFTR channels that are not functional in CF cardiac muscle.

Activation of CFTR by Recombinant β₁-Adrenoceptors—
CFTR channels are known to activate under intracellular cAMP (18, 19) or cGMP elevation (20, 21). The sensitivity of recombinant CFTR protein to intracellular cAMP and cGMP was investigated in A549 cells intranuclearly injected with a CFTR encoding plasmid (100 μg/ml) and monitored with the SPQ fluorescence assay. A549 cells were chosen because this cell line was previously demonstrated to lack endogenous CFTR protein (22). Under base-line conditions, cells injected with plasmids alone or containing the CFTR expression cassette exhibited a low permeability to halide (Fig. 2A and Fig. 3, left panel). Cells expressing CFTR but not cells injected with the plasmid lacking the insert displayed a ~6-fold increase in the rate of SPQ dequenching upon application of the cAMP-stimulating mixture (Fig. 2A and Fig. 3, left panel). Similarly, in cells expressing CFTR, the rate of SPQ dequenching was increased approximately 3-fold by pre-incubation with 500 μM CPT-cGMP (Fig. 2, B and C). As shown in Fig. 2C, the effect of cGMP stimulation was partially reversible upon CPT-cGMP washout. From this first set of experiments, we concluded that recombinant CFTR proteins produced by intranuclear injection of CFTR plasmid were sensitive to stimulation through both the cAMP- and cGMP-dependent pathways.

In a second set of experiments, various cell lines co-expressing either recombinant or endogenous CFTR and recombinant β3-adrenoceptor were investigated. In A549 cells injected with β3-adrenoceptor isoform A alone, the base-line membrane permeability to halide was not different from cells injected with the plasmid lacking the insert (Fig. 3, left panel) either in the absence or presence of CAMP. Surprisingly, cells co-injected with CFTR plus the A or the C isoform of β3-adrenoceptor exhibited an 8–10-fold increase in the base-line that was not further increased upon cAMP stimulation (Fig. 3, left panel). To ensure that this effect was related to β3-adrenoceptor expression, cells were co-injected with CFTR cDNA and with a cDNA encoding a K+ channel (ROMK2). In these cells, pbase line was similar to that in control cells and markedly increased in response to cAMP elevation. In cells expressing the mutated ΔF508-CFTR protein, there was no increase in pbase line related to co-expression with β3-adrenoceptor A or C isoforms. As expected, ΔF508-CFTR proteins were not sensitive to CAMP even in the presence of the β3-adrenoceptor (Fig. 3, left panel). Similar results were also obtained in COS-7 cells injected (not illustrated) or transfected (Fig. 3, middle panel) with CFTR and β3-adrenoceptor plasmids. In transfected COS-7 cells, pbase line increased about 3-fold in cells co-expressing CFTR and either the A or C isoform of β3-adrenoceptor. This effect was not observed in cells expressing CFTR or β3-adrenoceptor alone. In COS-7 cells co-transfected with CFTR and the A or C isoform of β3-adrenoceptor, cAMP stimulation produced a small albeit significant increase in halide permeability (Fig. 3, middle panel). T84 cells endogenously express the CFTR protein (23). Again, T84 cells injected with the A isoform of β3-adrenoceptor cDNA exhibited a 2-fold increase in pbase line as compared with noninjected cells (Fig. 3, right panel). This set of experiments shows that recombinant β3-adrenoceptor activates both endogenous and recombinant CFTR irrespectively to the transfection method used.

Patch-clamp experiments were performed in cells co-expressing β3-adrenoceptor and CFTR to further characterize the halide conductance responsible for the increased membrane permeability. A549 cells co-expressing CFTR and the A isoform of β3-adrenoceptor displayed a high amplitude time-independent Cl− current in the absence of CAMP stimulation (Fig. 4). This Cl− current persisted in the presence of CAMP, and the amplitude of Cl− current recorded under CAMP stimulation in cells expressing CFTR alone. On average, in the absence of CAMP stimulation, the current amplitude at +60 mV was 25.2 ± 5.4 pA/PF (n = 12) in cells injected with CFTR plus β3-adrenoceptor but
only 8.1 ± 3.1 pA/pF (n = 11) in cells injected with CFTR alone (p < 0.05). These results confirmed that the increase in p_base line in cells co-expressing CFTR and β3-adrenoceptors was related to the activation of CFTR chloride current in the absence of cAMP stimulation.

Variable Expression Levels of CFTR and β3-Adrenoceptors—To modulate the effects of β3-adrenoceptor on CFTR activity, the levels of expression of CFTR and β3-adrenoceptor were varied independently. In A549 cells, varying the level of CFTR expression in the absence of β3-adrenoceptor expression did not modify the base-line permeability to halide (Fig. 5, left panel). In contrast, in the presence of a constant expression level of β3-adrenoceptor isoform A (100 μg/ml), p_base line increased as the CFTR plasmid concentration in the injected medium increased from 3 to 100 μg/ml (Fig. 5, right panel).

In another set of experiments, we varied the injected concentration of β3-adrenoceptor isoform A plasmid from 0.03 to 3 μg/ml in the presence of a constant CFTR concentration (30 μg/ml). In these cells, β3-adrenoceptors were selectively activated using 10 μM isoproterenol, a nonselective β-adrenoceptor agonist, in the presence of 10 μM nadolol, a β1- and β2-adrenoceptor antagonist (24). It is noteworthy that cells expressing CFTR alone were not responsive to isoproterenol in the presence of nadolol (data not shown), suggesting that A549 cells lack endogenous β3-adrenoceptors. As the expression level of β3-adrenoceptor isoform A was increased, p_base line increased, the effects of β3-adrenoceptor stimulation with isoproterenol plus nadolol were progressively reduced, and the effects of the cAMP-activating mixture were also reduced (Fig. 6). So, at a low injected β3-adrenoceptor cDNA concentration (i.e. 0.1 μg/ml), the base-line halide permeability was not different from control cells but increased 3–4-fold upon β3-adrenoceptor stim-

FIG. 3. CFTR activity in the presence of recombinant β3-adrenoceptor in A549, COS-7, and T84 cells assayed using the SPQ fluorescent probe. Cells were either injected (A549 cells, left panel; T84 cells, right panel) or transfected (COS-7 cells, middle panel) with various mixtures of plasmids as indicated on the abscissa: CFTR cDNA (CFTR +), mutated ΔF508-CFTR cDNA (ΔF508-CFTR), plasmid lacking the insert (mock), the A or C isoform of β3-adrenoceptor cDNAs (βA and βC), and K+ channel cDNA (ROMK). For cDNA injection experiments, each plasmid was used at 100 μg/ml, except the plasmid lacking the insert (mock; 130 μg/ml). For transfection, CFTR cDNA was used at 2 μg/coverslip and β3-adrenoceptor cDNAs at 1 μg/coverslip. A green fluorescent protein plasmid was also added so as to obtain a final amount of 4 μg of plasmid/coverslip. 24 h post-injection or post-transfection, the membrane permeability to halide (p in min⁻¹) was measured under base line (open columns) and under application of cAMP-stimulating mixture (filled columns). Data are the means ± S.E. of 3–42 different A549 cells, 24–44 COS-7 cells, and 14–27 T84 cells. A paired Student's t test compares the p value in the presence of cAMP to the p value under base-line conditions. An unpaired Student's t test compares the p_base line value to the p_base line value in cells expressing CFTR alone. **, p < 0.01; ***, p < 0.001.

FIG. 4. Whole cell chloride currents in the presence of recombinant β3-adrenoceptor in A549. A549 cells were injected with 100 μg/ml CFTR cDNA in the absence (top; CFTR +) or presence of β3-adrenoceptor isoform A cDNA (100 μg/ml; bottom; CFTR +/β3A +). 24 h post-injection, whole cell Cl− currents were recorded in the absence (baseline) and presence of the cAMP-stimulating mixture (cAMP). Superimposed current traces obtained by voltage steps, 20 mV increment, applied from −180 to +80 mV. Holding potential, −80 mV.
**β3-Adrenoceptors Control CFTR**

![Diagram](image-url)

**FIG. 5.** CFTR activity as a function of CFTR expression level in A549 cells. Membrane permeability to halide (p in min⁻¹) expressed as a function of the injected CFTR cDNA concentration (3–100 µg/ml) as indicated on the abscissa. The left panel (pA) shows membrane permeability values in cells co-injected with β₃-adrenoceptor isoform A whereas the right panel (pA+) shows membrane permeability values in cells co-injected with β₃-adrenoceptor isoform A cDNA (100 µg/ml). p values were measured under base line (open columns) and under application of the cAMP-stimulating mixture (filled columns). Data are the means ± S.E. of 9–56 different cells. A paired Student’s t test compares the p value in the presence of CAMP to the p value under base-line conditions. An unpaired Student’s t test compares the p_base line values between two experimental conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

![Diagram](image-url)

**FIG. 6.** CFTR activity as a function of β₃-adrenoceptor expression level in A549 cells. Membrane permeability to halide (p in min⁻¹) expressed as a function of the injected concentration of β₃-adrenoceptor isoform A cDNA (0.03–3 µg/ml) in the presence of CFTR cDNA (30 µg/ml). p was measured under base line (open columns), under β₃-adrenoceptor stimulation with 10 µM isoproterenol after a 10-min preincubation with 10 µM nadolol (iso + nadol; hatched columns), and under application of cAMP-stimulating mixture (filled columns). Data are the means ± S.E. of 9–56 different cells. A paired Student’s t test compares p values in the presence of β₃-adrenoceptor stimulation or cAMP to the p value under base-line conditions. An unpaired Student’s t test compares the p_base line values between two experimental conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

![Diagram](image-url)

**FIG. 7.** Effect of PKA inhibitors on CFTR regulation by β-adrenoceptor stimulation in A549 cells. Cells were co-transfected with CFTR cDNA (30 µg/ml) and β₃-adrenoceptor isoform A cDNA (0.1 µg/ml; +βA). Membrane permeability to halide (p in min⁻¹) measured under base line (open columns) and under β-adrenoceptor stimulation (hatched columns) with a nonselective β-adrenoceptor agonist (isoproterenol 0.1 µM; Iso, left panel) or with a full β₁-adrenoceptor agonist (SR 59811 1 µM; SR, middle panel) or with a partial β₂-adrenoceptor agonist (CGP 12177 1 µM; CGP, right panel), in the absence or presence of PKA inhibitors (100 µM Rp-8-Br-cAMPS and 100 µM Rp-8-CPT-cAMPS). Data are the means ± S.E. of 21–109 different cells. A paired Student’s t test compares the p value in the presence of β-adrenoceptor stimulation to the p value under base-line conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Effect of PKA inhibitors on CFTR regulation by β-adrenoceptor stimulation**

In another set of experiments, we determined whether the PKA activation was involved in CFTR regulation by β-adrenoceptor stimulation, A549 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). Rp-8-Br-cAMPS and Rp-8-CPT-cAMPS are specific for PKA type I and II, respectively (26). PKA-dependent CFTR stimulation by isoproterenol (for review see Ref. 27) was used as a control experiment to check efficient PKA inhibition under our experimental conditions. In A549 cells injected with CFTR cDNA alone, isoproterenol, through a β₁- and/or β₃-adrenoceptor agonist (SR 59811 1 µM; SR; middle panel) or with a partial β₂-adrenoceptor agonist (CGP 12177 1 µM; CGP, right panel), in the absence or presence of PKA inhibitors (100 µM Rp-8-Br-cAMPS and 100 µM Rp-8-CPT-cAMPS). Data are the means ± S.E. of 21–109 different cells. A paired Student’s t test compares the p value in the presence of β-adrenoceptor stimulation to the p value under base-line conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Lack of PKA Involvement in CFTR Activation by β₃-Adrenoceptors**—In an attempt to determine whether the PKA activation was involved in CFTR regulation by β₃-adrenoceptor stimulation, A549 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). Rp-8-Br-cAMPS and Rp-8-CPT-cAMPS are specific for PKA type I and II, respectively (26). PKA-dependent CFTR stimulation by isoproterenol (for review see Ref. 27) was used as a control experiment to check efficient PKA inhibition under our experimental conditions. In A549 cells injected with CFTR cDNA alone, isoproterenol, through a β₁- and/or β₃-adrenoceptor agonist (SR 59811 1 µM; SR; middle panel) or with a partial β₂-adrenoceptor agonist (CGP 12177 1 µM; CGP, right panel), in the absence or presence of PKA inhibitors (100 µM Rp-8-Br-cAMPS and 100 µM Rp-8-CPT-cAMPS). Data are the means ± S.E. of 21–109 different cells. A paired Student’s t test compares the p value in the presence of β-adrenoceptor stimulation to the p value under base-line conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

In another set of experiments, we determined whether the basal activation of CFTR by a high level β₃-adrenoceptor expression was dependent on an increase in intracellular cyclic nucleotides. To test this hypothesis, intracellular cAMP and cGMP levels were measured in COS-7 cells co-transfected with CFTR and β₃-adrenoceptor isoform A. COS-7 cells were used for these experiments because transfection of A549 cells using various synthetic vectors led to a low number of cells expressing the transgene as assessed with a green fluorescent protein reporter. COS-7 cells were transfected under the same experimental conditions as used for functional CFTR tests (Fig. 3, middle panel). 24 h post-transfection, intracellular cAMP levels were determined under base line and after 5 min-incubation with 10 µM forskolin. As illustrated in Fig. 8A, base-line cAMP levels were not significantly modified by expression of either the A or C isoforms of β₃-adrenoceptor in cells expressing CFTR or not. As expected, pre-incubation with forskolin induced an elevation in cAMP level in every experimental situation. The
level of intracellular cGMP was also determined under base line and after a 20-min incubation with a cGMP analog, CPT-cGMP (500 μM; Fig. 8B). In cells expressing the CFTR protein and either the A or C isoforms of β₃-adrenoceptor, base-line cGMP concentration was not different from that of control cells. Comparison between results shown on Fig. 3 (middle panel) and Fig. 8, which were both obtained under the same transfection conditions, shows that β₃-adrenoceptor-mediated CFTR activation was not related to the activation of either the cAMP or cGMP pathways.

**DISCUSSION**

The present study suggests that β₃-adrenoceptors are functionally coupled to the CFTR protein. Interestingly, coupling between β₃-adrenoceptors and CFTR depends on the expression level of β₃-adrenoceptors: (i) expression of a low level of β₃-adrenoceptors induces CFTR activation in response to β₃-adrenoceptor agonists and (ii) expression of a high level of β₃-adrenoceptors induces permanent CFTR activation independently from β₃-adrenoceptor stimulation. In addition, we also show that functional coupling between CFTR and β₃-adrenoceptors occurs irrespectively to the β₃-adrenoceptor splice variants expressed in human tissues. Finally, our results show that regulation of CFTR by β₃-adrenoceptors does not imply activation of the cAMP/PKA pathway.

We have previously reported that intranuclear injection of large quantities of CFTR cDNA into mammalian cells produces hyper-expression of CFTR proteins with altered physiological properties inasmuch as hyper-expressed recombinant CFTR channels are permanently opened and not susceptible to cAMP stimulation (13). This phenomenon, which we attributed to CFTR clustering within the cell membrane, appears for various CFTR cDNA concentrations depending on the cell line. Accordingly, it may be argued that the increase in base-line permeability that we observed in cells co-expressing CFTR and β₃-adrenoceptors was caused by a comparable phenomenon. We judge this explanation unlikely for several reasons: (i) in A549 cells, permanent activation of hyper-expressed CFTR protein usually occurs for plasmid concentrations greater than 350 μg/ml (13), whereas in the present study, the injected concentration never exceeded 100 μg/ml, a concentration that did not lead to permanently activated CFTR channels in the absence of β₃-adrenoceptor co-expression; (ii) base-line activation of CFTR channels by recombinant β₃-adrenoceptors was observed in COS-7 cells co-transfected with CFTR and β₃-adrenoceptors, although permanent activation of CFTR channels was never observed in cells transfected with CFTR alone irrespectively to the quantity of cDNA used for cell transfection; thus, base-line activation of CFTR in the presence of β₃-adrenoceptors was unlikely to be caused by our intranuclear injection technique; (iii) furthermore, activation of CFTR at base line was not observed when β₃-adrenoceptor cDNA was replaced by a K⁺ channel cDNA inserted into the same plasmid and injected at the same concentration as β₃-adrenoceptor cDNA; and (iv) finally and most importantly, CFTR endogenously expressed in T84 cells was also susceptible to activation by β₃-adrenoceptors, suggesting that agonist-independent activation of CFTR can be observed for level of CFTR expression close to physiological ones.

Our results show that the effects of β₃-adrenoceptor expression on CFTR activity can be modulated by the density of the receptors within the cell membrane. Increasing the expression of β₃-adrenoceptor in the presence of a constant CFTR expression level dose-dependently increased base-line permeability and also concomitantly reduced the activating effects of β₃-agonists. The β₃-adrenoceptor belongs to the superfamily of G protein-coupled receptors (28). These receptors are known to exist in the cell membrane in two subpopulations: (i) an inactive subpopulation that requires agonist occupancy for coupling to G protein and (ii) a constitutively active subpopulation that can couple to G protein in the absence of agonist (29–31). An increase in the constitutively active subpopulation has been reported for mutated G protein-coupled receptors in which mutations induce a conformational change in the receptor that normally requires the binding of an agonist to occur (29, 32). Similarly, an increase in the constitutively active receptor subpopulation has been reported for receptors overexpressed at a high level. For example, overexpression of the wild-type β₃-adrenoceptor either in Chinese hamster ovary cells or in myocardial cells of transgenic mice produced an elevation of base-line adenylyl cyclase activity (29, 30, 33). Comparable behavior has been reported with the β₃-adrenoceptor so that basal adenylyl cyclase activity was raised with β₃-adrenoceptor density in Chinese hamster ovary cells (34). In the present study, when the expression level of β₃-adrenoceptors was high, CFTR was activated in the absence of β₃-adrenoceptor stimulation. In such conditions, intracellular cAMP and cGMP levels were close to normal and in any case much lower than the level required for CFTR activation as shown in Fig. 3 (middle panel). It could be hypothesized that overexpression of β₃-adrenoceptors led to a greater number of receptors in the active state, resulting in saturation in the β₃-adrenoceptor signaling capacity in the absence of agonist. Inversely, when the expression of β₃-adrenoceptors was lowered, CFTR was not activated under base-line conditions and became sensitive to agonists. Activation of CFTR by β₃-adrenoceptor agonists was not sensitive to PKA inhibitors, ruling out the involvement of the cAMP/PKA pathway. Activation of CFTR independently of the cAMP/PKA pathway has previously been reported for other receptors such as P₂Xₐ subtype of purinergic receptors (35) or mu-opioid receptors (36). Our results suggest that either another second messenger is implied in the coupling pathway between β₃-adreno-
ceptor and CRF or a direct interaction exists between the membrane receptor (or an associated G protein) and CRF. Although direct regulation of CRF channel protein by G proteins has not yet been reported, it has been shown that G_{sG}
protein modulates its vesicle trafficking and its delivery to the plasma membrane (37). As yet, two coupling pathways have been ascribed to β_{3}-adrenoceptors: (i) in adipose tissue and gastrointestinal tract, their stimulation produces an increase in cAMP levels (6, 7) and (ii) in human ventricle, the negative inotropic effects mediated by β_{3}-adrenoceptor agonists are associated with an increase in NO production and cGMP levels (8).

Clearly, the involvement of another mechanism leading to activation of CRF by β_{3}-adrenoceptors needs clarification.

The physiological relevance of our findings should be found in the various organs where β_{3}-adrenoceptors and CRF are co-expressed. In the human heart, β_{3}-adrenoceptor agonists produce a negative inotropic effect and a shortening in the action potential duration (5). Our results suggest that the action potential shortening produced by β_{3}-adrenoceptor agonists is caused by the activation of a CRF-related repolarizing chloride current that is nonfunctional in CF patients. β_{3}-Adrenoceptors and CRF are also endogenously co-expressed in other tissues such as airways (38, 39) and gallbladder (40) epithelia. In these tissues, β_{3}-adrenoceptors may modulate water and salt secretion through apical CRF channels.

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