Properties of CFTR activated by the xanthine derivative X-33 in human airway Calu-3 cells

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Received 20 January 2000; accepted in final form 8 August 2000

Cystic fibrosis transmembrane conductance regulator; chloride conductance; pharmacology

Cystic fibrosis (CF), the most common lethal autosomal recessive genetic disease, is caused by mutations of the CF gene, which normally encodes a multifunctional and integral membrane protein, the CF transmembrane conductance regulator (CFTR) (35). The native protein is a Cl\(^{-}\) channel located in the apical membrane of epithelial cells, where it mediates Cl\(^{-}\) trans-epithelial transport (for review see Ref. 25). CFTR mutations lead to an impaired or absent Cl\(^{-}\) conductance (13, 25), which generates defective Cl\(^{-}\) transport across the epithelium and perturbs the quantity and composition of epithelial fluids. This results in the manifestations of the disease, which include airway obstruction and infection, pancreatic failure, male infertility, and elevated levels of salt in sweat (for review see Ref. 23).

When the CFTR channel binds ATP at nucleotide binding domains and is phosphorylated at multiple sites within the R domain by cAMP-dependent kinases, it opens and generates a Cl\(^{-}\) flux (25). CFTR, mainly physiologically regulated by processes increasing intracellular cAMP, can also be stimulated by cAMP-independent pathways. Compounds such as p-bromotetramisole or levamisole (4, 6), genistein (29), 5-chloro-2-hydroxyphenyl-1,3-dihydro-2H-benzimidazol-2-one (NS-004) (15, 22), 1-ethyl-2-benzimidazolinone (1-EBIO) (15), 6-hydroxy-10-chlorobenzo[c]quinolizinium (MPB-07), and 6-hydroxy-7-chlorobenzo[c]quinolizinium (MPB-27) (5) have been proposed as new CFTR activators. All these molecules activate CFTR without altering the cell cAMP content. These results suggest other mechanisms of activation, such as the inhibition of endogenous CFTR-associated phosphatases (3, 4) or perhaps the direct binding to nucleotide binding fold (NBF) 1 or/and 2. Indeed, genistein has been shown to directly bind to NBF-2 and to compete with ATP binding on that site (34).

One goal of our group is to design pharmacological tools for research study and therapeutic application in...
CF. We recently showed that the use of synthetic xanthines may be important in discovering potent activators of the wild-type CFTR in Chinese hamster ovary (CHO) transfected cells by using iodide efflux and cell-attached patch-clamp experiments (9). Calu-3 cells, which are derived from a pulmonary adenocarcinoma, express high levels of CFTR mRNA and protein (21, 38) and secrete a Ca2+-dependent Cl− (38) and bicarbonate (16) fluid. The preponderance of CFTR channels in Calu-3 cells allowed further examination of channel properties in a well-differentiated epithelial cell. Moreover, Calu-3 cells resemble serous gland cells (21, 38), which have been identified as critical components in mucosal defenses and are implicated in CF lung disease (20, 43) and, therefore, are a relevant model to test the efficacy of CFTR activators.

We first describe in vitro phosphorylation and immunolocalization of CFTR on Calu-3 cells and report experiments using iodide efflux, whole cell recordings, and short-circuit techniques showing the effects of xanthine derivatives, including a novel compound obtained by chemical synthesis, 3,7-dimethyl-1-isobutylxanthine (X-33).

**METHODS**

**Cell culture.** Native CHO cells [CFTR(−) CHO] or CHO cells stably transfected with pNUT vector containing wild-type CFTR [CFTR(+) CHO] were provided by J. R. Riordan and X.-B. Chang (Scottsdale, AZ) (40). Cells cultured at 37°C in 5% CO2 were maintained in α-MEM containing 7% fetal bovine serum, 0.5% 1-glutamine, 0.5% antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin), and 100 μM methotrexate, as described previously in detail (4, 40). Calu-3, a cell line of human pulmonary origin (38), was cultured at 37°C in 5% CO2 and maintained in DMEM-Ham’s F-12 (1:1) nutritive medium (Gibco, Paisley, UK) and 8% fetal calf serum. The preponderance of CFTR expression in these cells (21, 38) and secretion of a cAMP-dependent Cl− flux indicates that they resemble serous gland cells (38, 43), which have been identified as critical components in mucosal defenses and are implicated in CF lung disease (20, 43) and, therefore, are a relevant model to test the efficacy of CFTR activators.

**Cell membrane preparation.** Calu-3, CFTR(−) CHO, and CFTR(+) CHO cells were removed from cell culture flasks by EDTA treatment and washed several times in Ca2+− and Mg2+−free PBS. Cell membranes were obtained as previously described (36) by lysing whole cells in Tris buffer (20 mM Tris-HCl, pH 7.45, 10 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1 mM dithiothreitol, 0.2 mg/ml benzamidine, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin, and leupeptin) for 15 min on ice. Nuclear and cell debris were removed by microcentrifugation (13,000 g) for 15 min at 4°C. The supernatant (50 μl, −4 mg protein/ml) was incubated with affinity-purified CFTR antibody (80 μg/ml) for 90 min at 4°C, and the antibody-CFTR complex was precipitated with Pansorbin (10% suspension of Staphylococcus aureus cells prewashed in RIPA buffer). The precipitate was washed, resuspended in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mg/ml BSA), and phosphorylated in vitro (60 min at 37°C) using the catalytic subunit of protein kinase A (PKA, 75 nM) and 10 μCi of [γ-32P]ATP. Phosphorylation was terminated by addition of RIPA buffer, and after several washes the immune complex was dissociated by solubilization in electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 5% SDS, 25% sucrose, 5% 2-mercaptoethanol) for 15 min at 37°C. Samples were subjected to SDS-PAGE on 7% separating gel and then Coomassie blue staining. Gels were dried and autoradiographed overnight using Hyperfilm-MP (Amersham). Lanes from the autoradiographs were scanned by densitometry (model GS-670, Bio-Rad) using Molecular Analyst software.

**Immunolocalization of CFTR.** Calu-3 cells, grown on coverslips to ~80% confluence, were fixed for 5 min at −20°C in 5% acetic acid in ethanol. All subsequent steps were carried out in PBS containing 0.1% fish gelatin and 1% BSA at room temperature. Cells were permeabilized with 0.2% (vol/vol) Triton X-100 for 20 min, and nonspecific protein binding sites were blocked by incubation first with 50 mM glycine for 30 min and then with 10% normal goat serum for 1 h. Cells were incubation with primary antibody (1:100 dilution) for 18 h at 4°C, washed three times for 15 min each, and incubated with secondary antibody conjugated with FITC (anti-rabbit IgG for CFTR; anti-mouse IgG for CD59) for 1 h and then washed three times. Slides were then mounted in Fluorosave Reagent (Calbiochem, La Jolla, CA). Fluorescence was detected using confocal laser scanning microscopy on a Zeiss Fluovert FU microscope (Leica, Germany) fitted with a TCS4D scanner (Leica). Confocal images were collected at a magnification of ×100 under oil immersion and displayed as Kalman averages on a 512 × 512-pixel 72-dpi screen. Images were

of conjugates (100–200 μg/ml), emulsified in Freund’s adjuvant, intradermally into rabbits. The antisera were affinity purified using peptide coupled to CH-Sepharose 4B (Pharmacia, Sweden), with elution of antibody fractions in 0.1 M glycine-HCl, pH 2.5. The antibody to CD59 (BRC1229) was obtained from the National Blood Service (Bristol, UK).

**Electrophoresis and immunoblotting.** SDS-PAGE of solubilized membrane proteins (50 μg) was performed by the method of Laemmli (32), with a 7% separating gel. After electrophoresis, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane at 50 V for 90 min at 4°C in a 25 mM Tris-HCl-0.7 M glycine buffer. Unreactive sites on the PVDF membrane were blocked with nonfat dry milk. The PVDF membrane was then incubated for 1 h at room temperature with a primary antibody raised against the COOH terminus of CFTR (Genzyme) at 0.4 μg/ml. After several washes, the membrane was incubated with a secondary antibody, horseradish peroxidase-labeled sheep antimouse IgG. Immunoreactive bands were visualized with a commercial enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Immunoprecipitation and phosphorylation of CFTR.** Calu-3, CFTR(−) CHO, and CFTR(+) CHO cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM iodoacetate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of chymostatin, pepstatin A, antipain, aprotinin, and leupeptin) for 30 min on ice. Nuclear and cell debris were removed by microcentrifugation (13,000 g) for 15 min at 4°C. The supernatant (50 μl, −4 mg protein/ml) was incubated with affinity-purified CFTR antibody (80 μg/ml) for 90 min at 4°C, and the antibody-CFTR complex was precipitated with Pansorbin (10% suspension of Staphylococcus aureus cells prewashed in RIPA buffer). The precipitate was washed, resuspended in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mg/ml BSA), and phosphorylated in vitro (60 min at 37°C) using the catalytic subunit of protein kinase A (PKA, 75 nM) and 10 μCi of [γ-32P]ATP. Phosphorylation was terminated by addition of RIPA buffer, and after several washes the immune complex was dissociated by solubilization in electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 5% SDS, 25% sucrose, 5% 2-mercaptoethanol) for 15 min at 37°C. Samples were subjected to SDS-PAGE on 7% separating gel and then Coomassie blue staining. Gels were dried and autoradiographed overnight using Hyperfilm-MP (Amersham). Lanes from the autographs were scanned by densitometry (model GS-670, Bio-Rad) using Molecular Analyst software.

**Antibodies.** For immunoblotting, a monoclonal antibody raised against the COOH terminus of CFTR was obtained from Genzyme (Cambridge, MA). For immunoprecipitation and immunolocalization studies, an antibody was raised against a peptide consisting of the 23 COOH-terminal amino acids of CFTR, as previously described for a CFTR antibody directed at the first nucleotide binding domain region (33). Briefly, the peptide was synthesized and coupled to keyhole limpet hemagglutinin (10 μg peptide/8 μg keyhole limpet hemagglutinin; Cambridge Research Biochemicals, Northwich, Cheshire, UK), and antisera were prepared by injection
processed using Scanware software (Leica), and image manipulation was performed using Corel Photopaint.

**AMP measurement.** Calu-3 cells were incubated in the presence or absence of test compounds. After a 5-min incubation period at 37°C, the reaction was stopped by addition of 250 μl of 12% TCA at 4°C. An RIA kit (RIANEN, NEN Life Science Products) was used to determine AMP levels.

**Iodide efflux experiments.** CFTR Cl⁻ channel activity was assayed by measuring the rate of iodide (125I⁻) efflux, as previously described (5). All experiments were performed at 37°C. Calu-3 cells grown in 12-well plates were washed twice with 2 ml of efflux buffer containing (in mM) 137 NaCl, 4.4 KCl, 0.3 KH₂PO₄, 0.3 NaH₂PO₄, 4.2 NaHCO₃, 1.3 CaCl₂, 0.5 MgCl₂, 0.4 MgSO₄, 5.6 glucose, and 10 HEPES, pH 7.5. Cells were then incubated in efflux medium containing 1 μM KI (1 μCi Na¹²⁵I/ml; NEN, Boston, MA) for 1 h at 37°C to permit the iodide to reach equilibrium. Cells were washed with efflux medium. After 1 min, the medium was removed and quickly replaced by 1 ml of the same medium. This procedure was repeated every 1 min for 11 min. The first three aliquots were used to establish a stable baseline in the presence or absence of test compounds. After a 5-min incubation period at 37°C, the reaction was stopped by addition of 2 ml of a Krebs bicarbonate solution containing (in mM) 126 NaCl, 0.4 KH₂PO₄, 2.1 K₂HPO₄, 1 MgSO₄, 1 CaCl₂, 24 NaHCO₃, 10 glucose, and 0.04 phenol red. During the experiments, this solution was kept at 37°C and continuously bubbled with 5% CO₂-95% air. The epithelium was short-circuited with a voltage clamp (model 558-C5, Dept. of Bioengineering, University of Iowa) connected to apical and basolateral chambers with Ag-AgCl electrodes. The potential difference and the fluid resistance between potential sensing electrodes were compensated. The Iₑₒ was recorded on a chart recorder (model L6512, Linseis). The Calu-3 cells occasionally showed some amiloride-sensitive current. To remove Na⁺ transport, all experiments were performed in the presence of 10 μM amiloride in the apical solution.

**Chemicals.** All products were obtained from Sigma Chemical (St. Louis, MO), except α-MEM and DMEM-Ham’s F-12 nutritive mix, which were acquired from Fisher and GIBCO BRL. TS-TM calix[4]arene (5,11,17,23-tetrasulfonato-25,26,27,28-tetramethoxy-calix[4]arene) was a generous gift of Drs. Singh and Bridges (University of Alabama at Birmingham).

**RESULTS**

**Expression and in vitro phosphorylation of CFTR in CHO and Calu-3 cells.** Calu-3 is an airway epithelial cell line that has retained most of the characteristics of serous gland stem cells (38). CFTR expression was analyzed using SDS-PAGE and immunoblotting. Results from Calu-3, CFTR(+), CHO, and CFTR(−) CHO cells are shown in Fig. 1A. In Calu-3 cells, the major CFTR form was a 175-kDa protein, as determined by molecular mass standard, which appreciatively comigrates with permanently expressed CFTR protein in CHO cells (Fig. 1A, lane 2). The smaller 145-kDa protein expressed in CFTR(+) CHO cells was visually absent in Calu-3 cells. No immunoreactivity was detected in CFTR(−) CHO cells (Fig. 1A, lane 3). As previously described (10), the 175-kDa protein represents the mature, fully glycosylated CFTR and the 145-kDa protein represents the immature or core-glycosylated CFTR. In addition, the upper band migrating at 175 kDa coincided with the phosphorylated band obtained by CFTR immunoprecipitation followed by phosphorylation of immunoprecipitates by the catalytic subunit of PKA in Calu-3 and CFTR(+) CHO cells (Fig. 1B, lanes 1 and 2).

**CFTR is in the apical membrane of Calu-3 cells.** CFTR was shown to have a predominantly apical location (Fig. 2A). It showed the same pattern of localization (Fig. 2B) as the GPI-anchored apical membrane protein CD59 (14). This was seen in x-y scans as a...
patch of immunofluorescence in the first confocal section (<1 μm from the apical surface). The labeling appeared as a ring of fluorescence at 3 μm from the apical surface and was not detectable 6–8 μm into the cell, thus having characteristics of an apical location. Specificity of CFTR immunofluorescence was shown by the demonstration that it was abolished by preabsorption of antibody with COOH-terminal peptide (Fig. 2C). Figure 2, A, d and B, d, show the side view (x-z scans) of CFTR and CD59 immunofluorescence, which also clearly indicates an apical location for both proteins.

Stimulation of CFTR-mediated iodide efflux by xanthine derivatives in Calu-3 cells. We examined the effects of IBMX and X-33 on Calu-3 cells. IBMX and X-33 were able to generate an iodide efflux significantly different (P < 0.0001) from the control conditions, i.e., in the absence of agonist (Fig. 3A). Addition of 250 μM IBMX or 250 μM X-33 increased the peak rate of iodide efflux from 0.09 ± 0.01 (n = 12) to 0.19 ± 0.01 (n = 24) and 0.15 ± 0.01 (n = 16), respectively (Fig. 3A). Caffeine (1,3,7-trimethylxanthine) at 250 μM was ineffective in stimulating an efflux from Calu-3 cells (data not shown). 8-Cyclopentyl-1,3-dipropylxanthine (CPX) was also applied at three different concentrations (10 nM, 10 μM, and 250 μM), but no significant iodide efflux was observed (data not shown). Forskolin (5 μM, n = 4) stimulates CFTR, as shown by the increase of peak rates (Fig. 3B). This forskolin-induced iodide efflux was strongly inhibited by 100 μM glibenclamide (n = 4; Fig. 3B) but was not affected by the outwardly rectifying Cl channel inhibitor (39) TS-TM calix[4]arene (200 nM, n = 4; Fig. 3B). The average dose-response relationships for IBMX and X-33 are presented in Fig. 3, C and D, respectively. These curves were obtained by normalizing the mean peak rates at various xanthine concentrations to that obtained with the saturating concentration. The EC50 values for IBMX and X-33 were 45 and 100 μM, respectively.

cAMP levels. We tested the possibility that activation by X-33 might be due to elevation of cAMP. In resting Calu-3 cells, the cellular cAMP content was 0.55 ± 0.05 pmol cAMP/well (n = 6; Fig. 3E). As expected, forskolin (5 μM) increased the cAMP level measured after 5 min (7.14 ± 0.71 pmol cAMP/well, n = 6; Fig. 3E). In contrast, the corresponding cAMP level determined in the presence of xanthine derivatives X-33 (250 μM, n = 6), CPX (10 nM, n = 3), and caffeine (250 μM, n = 3) was not increased compared with the basal level (Fig. 3E). These results argue against a role of cAMP in mediating the effect of X-33 on CFTR. IBMX (250 μM, n = 6) induced a weak elevation of cAMP content, significantly different from the basal level (P < 0.05; Fig. 3E).

Activation of CFTR current by the xanthine derivative X-33. It is established that Calu-3 cells are well polarized in culture (39), and we showed in Fig. 2 that CFTR is apically located. The iodide efflux data were completed by whole cell recordings to characterize the currents in Calu-3 cells. Figure 4 presents typical whole cell currents and associated current-voltage (I-V) plots in the presence or absence of activator in the bath. In control patch-clamp experiments on Calu-3 cells (Fig. 4, A and C; n = 20), i.e., in the absence of any activator, no current was recorded. We first used forskolin to test for the presence of cAMP-dependent Cl channel. The addition of 5 μM forskolin (Fig. 4B) stimulated a time-independent, nonrectifying conductance typical of functional CFTR. The forskolin-activated Cl conductance (Fig. 4C) had a current density of 17 ± 3.9 pA/pF (n = 11) when measured at +60 mV. The increase was statistically significant from the basal current (P < 0.001).

To further prove that the forskolin-activated current was due to CFTR activity, we performed ion-substitution experiments. Replacing all except 8 mM of the Cl in the bath solution with an equimolar amount of iodide led to a shift of the reversal potential toward positive potentials, from −37 ± 0.3 to +28 ± 2 mV (n = 3) in the presence or absence of forskolin, indicating a larger permeability for Cl, i.e., a selectivity of Cl >> I, consistent with a CFTR channel (26, 40).
We then tested the effects of the two xanthine derivatives IBMX and X-33 (Fig. 4, B and C). Addition of 250 μM IBMX to the bath caused a statistically significant increase in CFTR current amplitude compared with basal current [when measured at +60 mV, basal = 2.4 ± 0.3 pA/pF (n = 20) and IBMX = 20.4 ± 4.2 pA/pF (n = 4); Fig. 4C]. In CFTR(-) CHO cells, forskolin, IBMX, or X-33 failed to activate any current (n = 5 for each, data not shown). Addition to the bath of 250 μM X-33 caused the activation of a time-independent, nonrectifying conductance in 11 of 20 (55%) Calu-3 cells (Fig. 4, B and C). These increases were significantly different from basal current [16.2 ± 2.5 pA/pF (n = 11) in the presence of X-33 and 2.4 ± 0.3 pA/pF (n = 20) in control conditions at +60 mV]. Overall, X-33 activates a Cl⁻ conductance with CFTR-like kinetics (time- and voltage-independent, linear I-V relationship) in Calu-3 cells similar to that obtained for CFTR(+) CHO cells (not shown).

We then quantified the relationship between the X-33 concentration and the activated current in whole cell membranes from Calu-3 cells. The effects of increasing concentrations of X-33 were observed in the presence of 200 nM TS-TM calix[4]arene, a specific blocker of outwardly rectifying Cl⁻ current (39). A time course of the X-33 concentration-dependent activation of current in a Calu-3 cell is shown in Fig. 5A. Figure 5B shows averaged I-V curves obtained for different concentrations of X-33 applied to the bath. A complete dose-response relationship (Fig. 5C) was obtained by normalizing mean current amplitudes at various X-33 concentrations to that obtained with 250 μM X-33 (n = 4 for each concentration). This concentration was chosen because it is likely to maximally activate the X-33-dependent current. Elevating the X-33 concentration from 50 to 100 μM increased significantly the CFTR current. A concentration >250 μM generated a saturating X-33-dependent current. The EC₅₀ was consistent with the iodide efflux data.

Effects of Cl⁻ current inhibitors on X-33-activated current. The experiments described above show that CFTR activated by X-33 (Fig. 5) or forskolin (Fig. 3B) was not sensitive to the outwardly rectifying Cl⁻ current blocker calixarene. To confirm that this Cl⁻ current was mediated by the CFTR Cl⁻ channel, we examined the effects of two other Cl⁻ channel blockers, the sulfonamide glibenclamide and the arylaminobenzoate diphenylamine-2-carboxylate (DPC), both known as CFTR inhibitors (37). Results of such experiments are illustrated in Fig. 6, which shows representative current traces, time course of current, and I-V relationships in Calu-3 cells. The linear and nonrectifying X-33-activated Cl⁻ current was reduced to the basal level in the presence of 100 μM glibenclamide [16.2 ± 2.5 pA/pF with X-33 (n = 11) and 2.7 ± 0.4 pA/pF with glibenclamide (n = 3) at +60 mV; Fig. 6, A and C]. A time course of the activation and inhibition of current from the same whole cell recording is shown in Fig.
Addition of X-33 (250 μM) after 3 min of recording increased current within 6 min. In the presence of X-33, addition of glibenclamide (100 μM) at 7 min inhibited the majority of the current in 3 min. Overall, glibenclamide inhibited ~83% of the current (Fig. 6D). A similar result was obtained in iodide efflux experiments with glibenclamide (Fig. 3B). We also examined the effects of DPC. As shown in Fig. 6, B and C, 500 μM DPC induced a rapid and voltage-independent inhibition of X-33-activated current (0.55 ± 0.15 pA/pF, n = 3, at +60 mV). The time course (Fig. 6B,C) shows that, in the presence of X-33, DPC completely inhibited the current in 2 min. The effect of DPC is partially and slowly reversed (Fig. 6B,C). The maximal inhibition was 97% (Fig. 6D). The fact that DPC inhibition was voltage-independent is rather surprising but could be
Fig. 4. Activation of CFTR current in Calu-3 cells. Representative current traces for CFTR are shown in 3 representative Calu-3 cells before (A) and after (B) application of activator: 5 μM forskolin, 250 μM IBMX, or 250 μM X-33. Step protocol consisted of 300-ms voltage steps from −80 to +80 mV from a holding potential of −40 mV. Dashed lines, zero-current level. Capacitances for the 3 cells are 47, 44, and 55 pF, respectively. C: current-voltage (I-V) curves (means ± SE) for CFTR current before (n = 20) and after application of forskolin (n = 11), IBMX (n = 4), and X-33 (n = 11).
explained by the high concentration (500 μM). Flufenamic acid (FFA, 500 μM, n = 3) was also found to inhibit forskolin- or X-33-activated Cl\(^{-}\) current (not shown).

**Isc measurement.** We have measured the effects of X-33 on transepithelial ion transport under Isc conditions. Indeed, in this cell preparation, increases in Isc are an indication of anion secretion (16). Application of X-33 (10–250 μM) in apical and basolateral solutions determined a fast increase of Isc. The response consisted in an initial peak that was followed by a sustained phase (Fig. 7A). At 100 μM, the peak and the sustained phase (measured 15 min after X-33 application) were 14.7 ± 0.6 and 10.4 ± 1.1 μA/cm² (n = 4), respectively. The current activated by X-33 was not blocked by DIDS (Fig. 7B). Similar results were obtained using iodide efflux and whole cell recordings (data not shown). On the contrary, glibenclamide strongly blocked (Fig. 7A) or prevented (Fig. 7C) the effect of X-33. The sensitivity to glibenclamide and not to DIDS again suggested that X-33 activates the cAMP-dependent Cl\(^{-}\) channel, i.e., CFTR, as observed.
using iodide efflux and whole cell patch-clamp techniques. Consistent with this assumption, X-33 was ineffective when applied after stimulation with 5 μM forskolin (Fig. 7D), a concentration at which the cAMP-dependent current is maximally activated.

We have tested three other xanthines, IBMX, CPX, and caffeine, to compare their effectiveness with that of X-33. On a qualitative basis, the biphasic time course of the I_{sc} activated by IBMX or caffeine was very similar to that of X-33 and forskolin (Fig. 8A). However, IBMX was more potent, since at 10 μM, a concentration at which X-33 and caffeine had a small effect, this xanthine elicited almost a maximal current increase (Fig. 8B). On the contrary, caffeine was less effective, since only a weak effect was obtained at 250 μM. CPX was also found to have no effect (10 nM and 10 μM) or a weak effect (250 μM), as observed in iodide efflux (data not shown).

**Outwardly rectifying Cl⁻ current in Calu-3 cells.** It is now clear that the activity of CFTR in Calu-3 cells is not affected by DIDS or TS-TM calix[4]arene, as reported here. During whole cell patch-clamp experiments, we sometimes recorded a DIDS- and TS-TM calix[4]arene-sensitive Cl⁻ current. Figure 9 shows representative whole cell current traces and corresponding I-V curves (Fig. 9E) observed in 10 of 43 (23%) Calu-3 cells. In those cells, for high positive pulses (i.e., greater than +60 mV), a small outwardly rectifying Cl⁻ current, which deactivates with time, was observed in the absence of forskolin (Fig. 9A).

Addition of 5 μM forskolin to the bath leads to the activation of a weakly rectifying linear Cl⁻ current (Fig. 9B), which was partly inhibited by 100 μM glibenclamide (Fig. 9C). The remaining glibenclamide-

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**Fig. 7.** Effect of X-33 on short-circuit current (I_{sc}) in Calu-3 cells. Time course of I_{sc} in 4 representative experiments (A–D) is shown. Time of application of X-33 (100 μM, apical and basolateral), glibenclamide (500 μM, apical and basolateral), DIDS (500 μM, apical), and forskolin (5 μM, apical and basolateral) is indicated by arrows.

**Fig. 8.** Xanthine-dependent I_{sc} increase in Calu-3 epithelia. A: representative effects of IBMX and caffeine at 100 μM. B: summary of xanthine effects at different concentrations. Values are means ± SE of the current measured at the peak from ≥4 experiments.

**Fig. 9.** A DIDS-sensitive Cl⁻ current is present in Calu-3 cells. Representative current traces in a Calu-3 cell in control conditions (A) and in the presence of 5 μM forskolin with (C and D) or without (B) inhibitors are shown. E: corresponding I-V curves of instantaneous current (arrows on current traces; n = 10 for basal and n = 3 for each other condition). Capacitance is 34 pF. Glibenclamide, 100 μM; DIDS, 200 μM.
insensitive current was outwardly rectifying, deactivates with time, and was abolished in the presence of external 200 μM DIDS (Fig. 9D). These data indicate that an outwardly rectifying Cl− channel is expressed in Calu-3 cells, which could be activated by a cAMP-independent mechanism. Haws et al. (26), indeed, described at a single-channel level the properties of an outwardly rectifying Cl− channel in Calu-3 cells. This channel may be responsible for the outwardly rectifying whole cell Cl− current we recorded here. Because DIDS also inhibits several Cl− transporters, we tested the effect of TS-TM calix[4]arene, a compound related to DIDS and a more potent blocker of outwardly rectifying Cl− channels (37, 39). In the experiment described in Fig. 10, an outwardly rectifying Cl− current was spontaneously activated (Fig. 10A). The addition of 200 nM TS-TM calix[4]arene completely and rapidly inhibited this outwardly rectifying Cl− current (Fig. 10B; n = 5). Then, in the continuous presence of TS-TM calix[4]arene, 5 μM forskolin activated a linear CFTR current (Fig. 10C). This experiment reveals that TS-TM calix[4]arene inhibited outwardly rectifying Cl−, but not CFTR, current in Calu-3 cells and further demonstrates that the cAMP-activated Cl− current in this cell is mainly, if not exclusively, due to CFTR. In the 20 cells tested for activation by X-33, 11 responded by a linear CFTR Cl− current but none by an outwardly rectifying Cl− current, which clearly indicates the specificity of the xanthine derivative for CFTR.

DISCUSSION

Since the first electrophysiological characterization of the Cl− channel CFTR, the protein product of the CF gene (35), studying its pharmacology has become possible and appears to be of high importance not only to understand its regulation and role in normal and CF epithelia but because our knowledge of the pharmacology of CFTR (for review see Ref. 37) may serve as a model for the general pharmacology of Cl− channels.

Calu-3 cells, which are derived from a pulmonary adenocarcinoma, express a high level of CFTR mRNA and protein (21, 38), show cAMP-dependent Cl− (38) and bicarbonate (16) secretions, and resemble serous gland cells (26), the major pulmonary cells implicated in CF lung disease (20). In this study we showed that CFTR is in the apical membrane and is phosphorylated by PKA. Another type of outwardly rectifying Cl− current was also identified but was unlikely to contribute to the cAMP-dependent Cl− current in this cell.

CFTR and non-CFTR Cl− currents in Calu-3 cells. Despite the description of the basic properties of CFTR channels in this cell line (26, 42), little was known about their whole cell characteristics and pharmacology. At a single-channel level, Haws et al. (26) characterized two types of Cl− channels in Calu-3 cells: CFTR and outwardly rectifying depolarization-induced Cl− channels. In cell-attached recordings, CFTR has a linear I-V relationship with a single conductance of 7.1 pS, is voltage independent, and is activated by cAMP-elevating agents. We showed in this report that the cAMP-activated iodide efflux and CFTR Cl− current were inhibited by glibenclamide, DPC, and FFA but not by DIDS and TS-TM calix[4]arene.

Non-CFTR channels were observed in 17% of unstimulated cell-attached patches and 8% of stimulated
excised patches in Calu-3 cells (26). An outwardly rectifying depolarization-induced Cl\(^{-}\) channel was observed in unstimulated patches of Calu-3 cells but not in cell-attached patches (26, 42). In 13% of excised patches exposed to ATP plus PKA, Haws et al. (26) recorded outwardly rectifying depolarization-induced plus CFTR channels. In good agreement with these data, we recorded outwardly rectifying Cl\(^{-}\) current in only 23% of the unstimulated Calu-3 cells but in 0% of stimulated cells studied here. Since this current is activated mainly in the absence of cAMP, this suggests that the outwardly rectifying Cl\(^{-}\) current is not directly under the control of a cAMP pathway. The pharmacology of outwardly rectifying Cl\(^{-}\) current is based on the inhibitory effect of stilbene derivatives such as DIDS, SITS, or 4,4′-dinitroso-stilbene-2,2′-disulfonate (39). Recently, TS-TM calix[4]arene, a compound related to DIDS and a more potent blocker of outwardly rectifying Cl\(^{-}\) channels, was developed (39). We have also shown here that DIDS or TS-TM calix[4]arene inhibited the outwardly rectifying Cl\(^{-}\) current.

**Xanthine as CFTR activator.** The story of xanthine derivatives as modulators of CFTR began in 1991, when Drumm et al. (17) demonstrated that wild-type CFTR, ΔF508 CFTR, and other mutants expressed in Xenopus oocytes could be activated by IBMX in a concentration-dependent manner, but in the presence of forskolin (17). Then, in the absence of forskolin, the methylxanthine drug IBMX by itself was shown to activate normal and mutated CFTR Cl\(^{-}\) channels in epithelia (3, 25, 26) and transfected cells (4, 37). IBMX, 1,3-dipropyl-7-methylxanthine, and the synthesized xanthines X-33 and X-32 stimulate CFTR in transfected CHO cells without altering ATP contents and with little effect on intracellular cAMP level (9; this study). Nanomolar concentrations of CPX were reported to increase \(^{36}\)Cl efflux in CFPAC-1 pancreatic cells (homoygous for the ΔF508 allele) (19). Moreover, Arispe et al. (1) found that CPX could activate wild-type CFTR in planar lipid bilayer after a modest exposure to PKA and ATP. However, CPX seems to be more selective for ΔF508 CFTR than for the wild-type. Cohen et al. (11) proposed that this agent activates CFTR by a direct interaction on NBF-1. However, some authors have not observed any acute activation of CFTR by CPX (9, 27, 31), whereas others have reported a potentiation of the response to forskolin on ΔF508 CFTR-expressing cells consistent with that obtained with IBMX (27, 28).

**Xanthine as CFTR activator in Calu-3 cells.** Having demonstrated, along with others, that CFTR Cl\(^{-}\) channel is the main cAMP-dependent apical Cl\(^{-}\) pathway in Calu-3 cells (26, 38; this study), we have investigated the effect of xanthine derivatives on CFTR. As expected, IBMX elicited a significant and concentration-dependent (EC\(_{50}~\sim~50~\mu M\)) iodide efflux and whole cell current with all the characteristics of CFTR-mediated Cl\(^{-}\) transport. The synthetic xanthine X-33 was found to be effective in Calu-3 cells by the use of three different techniques (iodide efflux, whole cell patch-clamp, and short-circuit measurements). The I-V relationship of the activation of Cl\(^{-}\) transport was linear and time independent in whole cell recordings, with an EC\(_{50}\) of ~100 ~μM. The X-33-activated iodide efflux, whole cell current, and I\(_{sc}\) were remarkably DIDS insensitive and glibenclamide sensitive. More importantly, the effect of X-33 on Calu-3 cells was insensitive to calix[4]arene. This demonstrates that the outwardly rectifying Cl\(^{-}\) current found in this preparation is not affected by this agent or by forskolin. The other xanthines tested, caffeine and CPX, were poorly effective in activating a Cl\(^{-}\) current.

**Mechanisms of xanthine-dependent activation of CFTR.** Xanthine derivatives, depending on their structure, may act as inhibitors of phosphodiesterases (PDEs) (2) or phosphatases (3, 12) or as antagonists of adenosine receptors (7). Activation of CFTR channels using xanthine derivatives was initially attributed to inhibition of PDE activity, which would elevate cAMP by inhibiting its degradation. However, IBMX increased the maximal forskolin-dependent ΔF508-CFTR activity without increasing cellular cAMP, suggesting a cAMP-independent mechanism (28). A similar conclusion was drawn previously by Chappe et al. (9). They showed that, among the nonspecific PDE inhibitors IBMX, theophylline (1,3-dimethylxanthine), caffeine, and 8-cyclopentyltheophylline, only IBMX and theophylline were able to open CFTR (9). In this study, the xanthine derivative X-33 did not alter the intracellular cAMP content, as previously shown in CFTR(+) CHO cells (9). Moreover, the specific PDE inhibitors rolipram and milrinone failed to open CFTR channels in CFTR(+) CHO cells (9). For these reasons, it is reasonable to think that xanthine derivatives activate CFTR through a cAMP-independent mechanism. In particular, substituted xanthines IBMX and theophylline inhibit alkaline phosphatase (3, 12). Beq et al. (4) showed that these two compounds slowed the rundown of CFTR channel activity in excised membrane patches. Xanthine derivatives are also known to be antagonists of adenosine receptor A (7). Using pancreatic duct cells expressing the ΔF508 mutation (CFPAC cells), Eidelman et al. (19) initially reported the activation of Cl\(^{-}\) current by CPX, a potent A\(_1\) adenosine-receptor antagonist (19). No human A\(_1\) adenosine-receptor mRNA was later detected in these cells (30), excluding this receptor as a mediator of CPX-elicited Cl\(^{-}\) efflux. The authors then suggested that the action of CPX on ΔF508-CFPAC cells represents a novel site of action apparently unrelated to a known adenosine receptor. Casavola et al. (8) reported that CPX decreased intracellular pH, which would be linked to the inhibition of Na\(^+\)/H\(^+\) exchange. It is not known, however, whether the action of CPX on the Cl\(^{-}\) efflux may be correlated with the variation of intracellular pH or with some other intracellular mechanisms. More recently, Kunzelman et al. (31) reported that the CPX-induced Cl\(^{-}\) efflux observed in CF cells could not be attributed to a direct activation of ΔF508 but, on the contrary, may be due to a pH-dependent mechanism. Finally, a controversy appeared when other groups failed to reproduce the effect of CPX (27, 31). Some
authors showed a biphasic response to CPX, i.e., a stimulation for low concentrations (10–30 nM), whereas higher concentrations (100 nM–10 μM) caused inhibition of 36Cl efflux (19, 24, 30). In our experience (4, 9; this study), stimulation of CFTR by CPX has not been observed in CHO cells stably transfected with wild-type CFTR and in Calu-3 cells, whatever the concentration used (10 nM, 10 μM, and 250 μM).

**Structure-function study.** We are presently using a series of alkyl-substituted xanthine derivatives to study the effects on CFTR of chemical modifications of the xanthine skeleton (9). Previously, we showed a correlation between the potency of a series of 1,3,7-trialkylxanthine derivatives and the opening of the CFTR Cl⁻ channel (9). To understand the substitutions implicated in the activation of CFTR, caffeine was chosen in this study, because it differs from X-33 only at N-1 (methyl and isobutyl, respectively). This modification leads to a potent activator (X-33) and a far less active one (caffeine). The alkyl substitution at N-1 thus appears to be important for CFTR activation. However, the same modification (isobutyl) at N-3 produces a more potent activator (IBMX). So we can hypothesize that the presence of a long alkyl bond at N-1 generates agents with high potency on CFTR activation, but a second important site seems to be located at N-3. Although X-33 is about one-half as potent as IBMX, X-33 has no effect on the cell cAMP levels, and it is extremely important to increase the spectrum of compounds capable of elevating specifically the CFTR activity. Our data suggested that xanthines substituted at C-8 (CPX) failed to open CFTR in Calu-3 cells, which suggests that the general mechanism of CFTR opening by xanthines required alkyl substitution (at N-1 or N-3) and vacancy at C-8 in CHO and Calu-3 cells. The role of CFTR in the Cl⁻ conductance of several tissues (e.g., heart, nephron, or exocrine pancreas) may be clarified by the use of specific activators. For example, the cardiac CFTR channel has been recently shown to be activated by levamisole (18), a compound we have identified by its effects in pancreatic duct cells (3) and in CHO cells (4).

In conclusion, the pharmacology of CFTR is at the point where numerous families of chemicals of interest have been discovered, which opens a very exciting avenue toward the development of agents of high importance for the knowledge of the physiology of epithelia and other tissues, including the heart, and of the pathophysiology, including CF. Solving the structure-activity relationships of these chemicals will help build further steps toward those goals in the future.

The authors thank Ashvani Singh and Robert Bridges for the generous gift of calixarene.

This work was supported by postdoctoral fellowships to L. Bulteau and a thesis grant to R. Dérand from the Association Française de Lutte contre la Mucoviscidose, by institutional grants from Centre National de la Recherche Scientifique, and by Association Française de Lutte contre la Mucoviscidose Grant P-97003.

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