A_1 Receptor Antagonist 8-Cyclopentyl-1,3-dipropylxanthine Selectively Activates Chloride Efflux from Human Epithelial and Mouse Fibroblast Cell Lines Expressing the Cystic Fibrosis Transmembrane Regulator ΔF508 Mutation

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ABSTRACT: Cystic fibrosis is an autosomal recessive disorder affecting chloride transport in pancreas, lung, and other tissues, which is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR). The A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX) stimulates $^{36}Cl^-$ efflux from pancreatic CFPAC-1 cells which bear the ΔF508 genotype common to most cases of cystic fibrosis [Eidelman et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 5562−5566]. By contrast, correction of the cystic fibrosis defect by retrovirus-mediated gene transfer renders the resulting CFPAC-PLJ-CFTR cells insensitive to CPX. We now report that CPX also activates chloride efflux from the CF tracheal epithelial cell line IB3-1 bearing a ΔF508 allele, but not if the IB3-1 cells have been repaired by transfection of the wild-type CFTR gene. Similar results were obtained with recombinant NIH 3T3 cells, in which CPX activates $^{36}Cl^-$ efflux from cells expressing the CFTR(ΔF508) gene product but not from 3T3 cells expressing the wild-type CFTR. In all three cell types expressing CFTR(ΔF508), CPX was found to activate $^{36}Cl^-$ efflux in a dose-dependent manner over the concentration range of 1−30 nM and then gradually lose potency at higher CPX concentrations. Six CPX analogues, A_1 receptor antagonists of affinity similar to that of CPX, were found to be much less effective than CPX at activating $^{36}Cl^-$ efflux from CFPAC-1 cells. These included 2-thio-CPX. CPT (8-cyclopentyl-1,3-dimethylxanthine), 3,4-dehydro-CPX, 3-F-CPX, 3-I-CPX, and KW-3902 (8-noradamantyl-1,3-dipropylxanthine). We conclude from these studies that CPX can activate chloride efflux from CF epithelial cells in which the CFTR(ΔF508) genotype is *present* and the wild-type CFTR gene is *absent.* The presence of excess wild-type CFTR in repaired CFPAC-1 and 3T3-CFTR cells was verified by Western blot analysis. In addition, the nature of the gene transfer vehicle does not seem to be important for the loss of sensitivity to CPX. The fact that this relationship can also be demonstrated with mouse 3T3 cells indicates that the CPX effect is not exclusively dependent upon a human epithelial cell substrate. Finally, the comparative data obtained with a variety of selective A_1 antagonists lead us to question the hypothesis that the CPX effects on CF cells occur via interactions with a classical A_1 receptor. Alternative possibilities include either direct action of CPX upon the ΔF508 mutant of CFTR or action on a yet-to-be-characterized purine binding site common to both human and mouse cells.

Cystic fibrosis (CF) is an autosomal, recessive disease (Riordan et al., 1989; Rommens et al., 1989; Karem et al., 1989), which is manifest at the cellular level in pancreas, lung, and other affected tissues by a reduced cAMP-activated chloride efflux (Quinton, 1990; Boat et al., 1989) through a channel correlated with expression of some or all of the cystic fibrosis transmembrane regulator (CFTR) gene product. Supporting data have come from studies in both natural and recombinant cells (Anderson et al., 1991a,b; Drumm et al., 1990) or planar lipid bilayers (Arispe et al., 1992; Bear et al., 1992). Recently, the A_1 receptor antagonist CPX (DPCPX; 8-cyclopentyl-1,3-dipropylxanthine) has been shown to stimulate chloride efflux from pancreatic CFPAC-1 cells, which bear the ΔF508 genotype common to most cases of cystic fibrosis (Eidelman et al., 1992). The CPX concentration leading to optimal activation of chloride efflux was in the range of 10−30 nM, as was the activity of one other A_1 receptor antagonist, xanthine amino congener (XAC; 8-[[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine). We noted, however, that these drugs had no influence on chloride efflux either from CFPAC cells repaired with the wild-type CFTR gene (CFPAC-PLJ-CFTR), or from a variety of human colonic epithelial cells lines bearing the wild-type CFTR, such as T-84 or HT-29. Given the low toxicity of CPX and its apparent selectivity for the ΔF508 genotype, relative to the wild-type CFTR, we proposed CPX as a candidate drug to treat the chloride permeability defect associated with cystic fibrosis.

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In pursuit of evaluating the therapeutic potential of CPX, we have extended our study to cells other than those derived from pancreatic tissue of CF patients. Preliminary studies on the cystic fibrosis cell line IB3, derived from the tracheal epithelium of a CF patient, indicated that this cell, too, was sensitive to CPX (Eidelman et al., 1992). In work to be described here we confirm this result and further show that IB3 cells repaired with the wild-type CFTR are rendered insensitive to CPX. We also found similar, differential effects of CPX on chloride efflux from mouse fibroblast 3T3 cells expressing either human CFTR or the human CFTR-(ΔF508) gene products. In sum, our data indicate that the activation of chloride efflux by CPX occurs in any of these cell types, so long as they possess the ΔF508 genotype but are not repaired with the wild-type gene. We further found that six other A_1 receptor-selective antagonists were considerably less efficacious than CPX.

MATERIALS AND METHODS

Cells. CFPAC-1, CFPAC-PLJ-CFTR, and CFPAC-PLJ cells (Drumm et al., 1990) were obtained from Dr. R. Frizzell, University of Alabama at Birmingham. IB3-1 and S9 cells have been previously described (Egan et al., 1992). NIH 3T3-CFTR and 3T3-CFTR-ΔF508 cultures were obtained from Dr. M. J. Welsh, University of Iowa (Anderson et al., 1991b). A random culture of NIH 3T3 cells was purchased from the American Type Culture Collection (ATCC, Bethesda, MD).

Cell Culture Methods. CFPAC cells were grown in Iscove's medium, supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 1% (w/v) glutamine. IB3 and S9 cells were grown in LHC-8 medium, supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 1% (w/v) glutamine. 3T3 cells were grown in Eagle's minimal essential medium (EMEM), supplemented with 10% adult bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 1% (w/v) glutamine. All culture materials were obtained from Biofluids, Rockville, MD, and the osmolarity was 310 mosM. Prior to flux experiments, cells were split and seeded at low density on Costar 24-well plates in the respective medium for each cell line. After 5 h, the medium was replaced and the attached cells were allowed to grow to confluency during a period of 48−72 h at 37 °C in 5% CO_2/95% air.

Measurement of ^{36}Cl Fluxes. Cells were loaded with $^{36}Cl^-$ as follows: Confluent cells were washed four times in their respective media. Following the last wash, 500 μL of medium containing approximately 1.4×10^8 cpm of $^{36}Cl^-$ (Amersham) was added to each well. Plates of cell were then incubated overnight at 37 °C in 5% CO_2/95% air to allow $^{36}Cl^-$ isotopic equilibration. The bicarbonate-free condition previously employed (Eidelman et al., 1992) was not found to be necessary. To initiate efflux experiments, appropriate concentrations of CPX or other xanthines were added to the cells and incubated for 15 min at 19 °C. The wells were then washed four times with ice-cold wash medium containing 150 mM sodium gluconate and 10 mM Na-HEPES, pH 7.4. The subsequent bicarbonate-free flux medium consisted of 150 mM sodium gluconate, 1.5 mM potassium gluconate, and 10 mM Na-HEPES, pH 7.4, with 100 μM bumetanide to inhibit possible efflux via the cotransporter and different concentrations of CPX or other compounds, as specified. Efflux assays were then performed exactly as described previously (Eidelman et al., 1992), except that the temperature was 19 °C rather than 21 °C, and time intervals were tailored to suit the kinetics of each type of cell. At the end of each flux experiment, the $^{36}Cl^-$ remaining in the cells was measured by solubilizing the residual fraction in 5% trichloroacetic acid (TCA). In some cases, the potassium concentration was raised by replacement of sodium gluconate with potassium gluconate. To verify that washout of extracellular label was complete, we added ^{3}[H]inulin to the cells and measured the extent of washout of external label. By the fourth wash of our standard protocol, the level of detectable tritium in the extracellular phase was decreased to essentially that of the background level.

In experiments to perform a compartmental analysis of chloride efflux, cells were loaded with ^{36}Cl for 4 h in a bicarbonate-free medium in a CO_2-free incubator at 37 °C. The cells were washed as above and then allowed to efflux into either a chloride-containing medium (Krebs solution) or a medium in which gluconate replaced chloride (see description above). In each case the experiments were performed at 19 °C in the absence of bicarbonate. For this analysis the total extracellular medium was totally replaced by fresh medium at each time point. The data were analyzed by fitting the data to

$$Cl_i(t) = Ae^{-kt} + B \quad (1)$$

where $Cl_i(t)$ is the intracellular amount of chloride at any time t, k is the efflux rate constant from the mobile compartment, A is the size of the mobile compartment, and B is the size of the immobile compartment. The value of $Cl_i(t)$ was calculated by subtracting the amount of chloride in the system. The latter is the sum of all the samples taken including the chloride released by trichloroacetic acid (5%) at the end of the experiment. The model shown in eq 1 was the best model to fit the data using up to five parameters. More complicated models such as (a) two exponentials and a constant or (b) one exponential, a linear component, and a constant gave, respectively, either a very low amplitude (<3%) for one of the exponents or a very slow rate for the linear component.

Gel Electrophoresis and Western Blots. Cells were prepared for analysis essentially as described by Breuer et al. (1992) with minor modifications. Briefly, cells grown to confluency in 25-mL flasks (Corning) were washed three times with ice-cold TBS (140 mM NaCl and 10 mM Tris-HCl, pH 7.3), and scraped using a cell scraper (Costar) into 0.6 mL of solubilization buffer (TBS containing 5 mM EDTA, 5 mM dithiothreitol, 1% Triton X-100, 0.5 mM PMSF, 25 μg/mL benzamidine, 10 μg/mL TPCK, 10 μg/mL TLCK, 1 μg/mL pepstatin, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 8 μg/mL bestatin). Undisrupted cells, nuclear debris, and detergent-insoluble material were removed by centrifugation at 10000*g* for 15 min at 4 °C. The supernatant solution was incubated for 10 min at 37 °C in SDS−PAGE sample buffer [1.25% SDS, 1.25 M urea, 25 mM dithiothreitol (DTT), 5% glycerol, 0.1% pyronin Y, and 125 mM Tris-HCl, pH 6.8). A small aliquot of each sample was retained for total protein determination, and samples containing *ca.* 50 μg of protein were electrophoresed on 6% acrylamide minigels (Novex) and transferred to nitrocellulose paper using a water-cooled Hoefer minielectrotransfer ap-

paratus (300 mA for 3 h at 5 °C). The quality of the transfer was assessed by staining with 0.5% Ponceau S in 1% acetic acid and washing in 1% acetic acid (Breuer et al., 1993). After the blot was blocked for 1 h at room temperature in m-PBS-T (6% nonfat dry milk in 150 mM NaCl, 10 mM sodium phosphate, and 0.05% Tween-20, pH 7.3), the blot was reacted with M3A7 monoclonal anti-CFTR antibody (a gift from Drs. N. Kartner and J. R. Riordan, Hospital for Sick Children, Toronto, Canada; Kartner et al., 1992) by overnight incubation at 4 °C. The localized antibody was detected using the enhanced chemiluminescence method (ECL kit, Amersham) for varying periods of exposure time as indicated in the text.

Chemicals and Chemical Analysis. CPX (8-cyclopentyl-1,3-dipropylxanthine) and CPT (8-cyclopentyl-1,3-dimethylxanthine) were obtained from RBI (Natick, MA). Other CPX analogues were synthesized as reported (Jacobson et al., 1988, 1989; Shimada et al., 1992). KW-3902 (8-noradamantyl-1,3-dipropylxanthine) was synthesized as follows: 3-Noradamantanecarboxylic acid was condensed with 5,6-diamino-1,3-dipropyluracil (Shimada et al., 1992) in dimethylformamide using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride as the coupling reagent. Saline was added and the crude product (6-amino-1,3-dipropyl-5-[[(noradamant-3-yl)carbonyl]amino[uracil) was isolated by extraction into ethyl acetate. Following evaporation, the residue was added to a mixture of 4 N NaOH and methanol (3:5) and heated to reflux for 15 min. Upon cooling and acidification with 4 N HCl, the final product was separated as a precipitate, purified by chromatography on a silica gel plate (ethyl acetate−petroleum ether 1:1) and then further purified by recrystallization from dimethylformamide/water (mp 186 °C).

Protein was determined by the BCA method using a kit from Pierce Chemicals. $^{36}Cl^-$ was measured by scintillation spectrometry on a Beckman LS9000. Osmotic strength was measured by freezing-point depression on an Osmette osmometer.

Data and Data Analysis. Each experiment presented was performed at least four times, and within each experiment each data point was computed from the average of efflux measurements performed on four separate wells. The vertical axis in each flux study (FA) represents the fraction of the radiolabeled chloride remaining within the cell at the given time. The calculated rate constants, presented in a bar graph format, were calculated by linear regression of log (FA) as a function of time and are presented as a percentage relative to the rate constants of control wells on the same plate. Data were handled with the Quatro-Pro 4.0 program on a 386 PC. The error bars are the SEM's of four experiments (i.e., n = 4).

RESULTS

Potassium Diffusion Potential Drives ^{36}Cl Efflux from CFPAC-1 Cells. Cystic fibrosis cells such as CFPAC-1 can be loaded with ^{36}Cl and the differential rate of chloride efflux can be used to evaluate the ameliorative influence of drugs such as CPX on the common CFTR mutation, ΔF508 (Eidelman et al., 1992). The driving force for the flux is presumed to be influenced by the potassium diffusion potential across the cell plasma membrane. To verify that this is indeed the mechanism for chloride efflux from CFPAC-1 cells, we analyzed chloride efflux from these cells

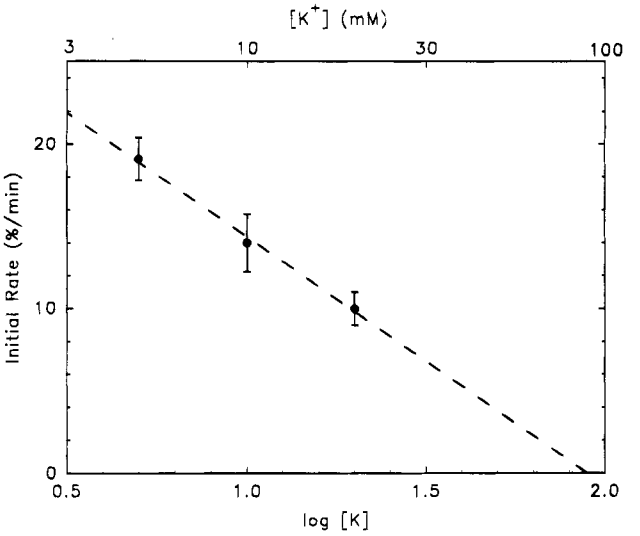

FIGURE 1: Effect of extracellular potassium concentration on ^{36}Cl efflux from pancreatic CFPAC-1 cells. Efflux of chloride from CFPAC-1 cells loaded with ^{36}Cl was allowed to proceed into a sodium gluconate medium containing either 5, 10, or 20 mM potassium gluconate. The initial rate of chloride efflux, estimated from the average rate during the first 2 min of the flux, is plotted against $[K]_{out}$ on a logarithmic scale. The line through the points intersects with the abscissa at $[K]_{out}$ = 84 ± 8 mM (SD, n = 4).

in the presence of increasing concentrations of K^+. In these and other experiments (Eidelman et al., 1992) a temperature of 19 °C was chosen to blunt the potential contributions of the two major transporters, the Cl^-/HCO_3^- exchanger and the $K^+/Na^+/2Cl^-$ cotransporter. As shown in Figure 1, the initial rate of efflux decreased with increasing extracellular potassium in a logarithmic fashion. We also found that the line through the points intersected the horizontal axis at $[K_{out}]$ = ca. 85 mM. This result conforms with the notion that as the external potassium concentration increases, the rate of chloride efflux approaches zero. These data also indicate that the chloride efflux is driven by the electrochemical potential and that no significant fraction of the efflux is independent of this specific driving force.

Compartmental Analysis of ^{36}Cl from CFPAC-1 Cells. A second assumption in our analysis has been that the $^{36}Cl^-$ efflux process is from a single intracellular compartment. To test this assumption, we analyzed the efflux process either in media containing physiologic chloride concentrations or in media where gluconate replaced chloride. As shown in Figure 2A, efflux of $^{36}Cl^-$ from CFPAC-1 cells into a chloride-containing medium occurred by a process which could be fit by an equation which describes one mobile compartment and one immobile compartment. The solid line in *Figure 2A* is the fit to this equation. The open symbols and dotted line in *Figure 2A* are data for cells in the presence of CPX, which we have found to activate chloride efflux from these cells. In *Figure 2B* we performed the same analysis for a system in which the medium contained gluconate in place of chloride. In this case the efflux of $^{36}Cl^-$ from both control and CPX-treated cells was similarly well fit by eq 1, although the CPX effect was slightly less robust.

The data in *Figure 2A,B* were further evaluated in terms of rate constants and initial rates. As shown in *Figure 2C*, the rate constant for efflux of ^{36}Cl from CPX-treated CFPAC-1 cells was nearly twice that of the control when the medium contained physiologic concentrations of chloride.

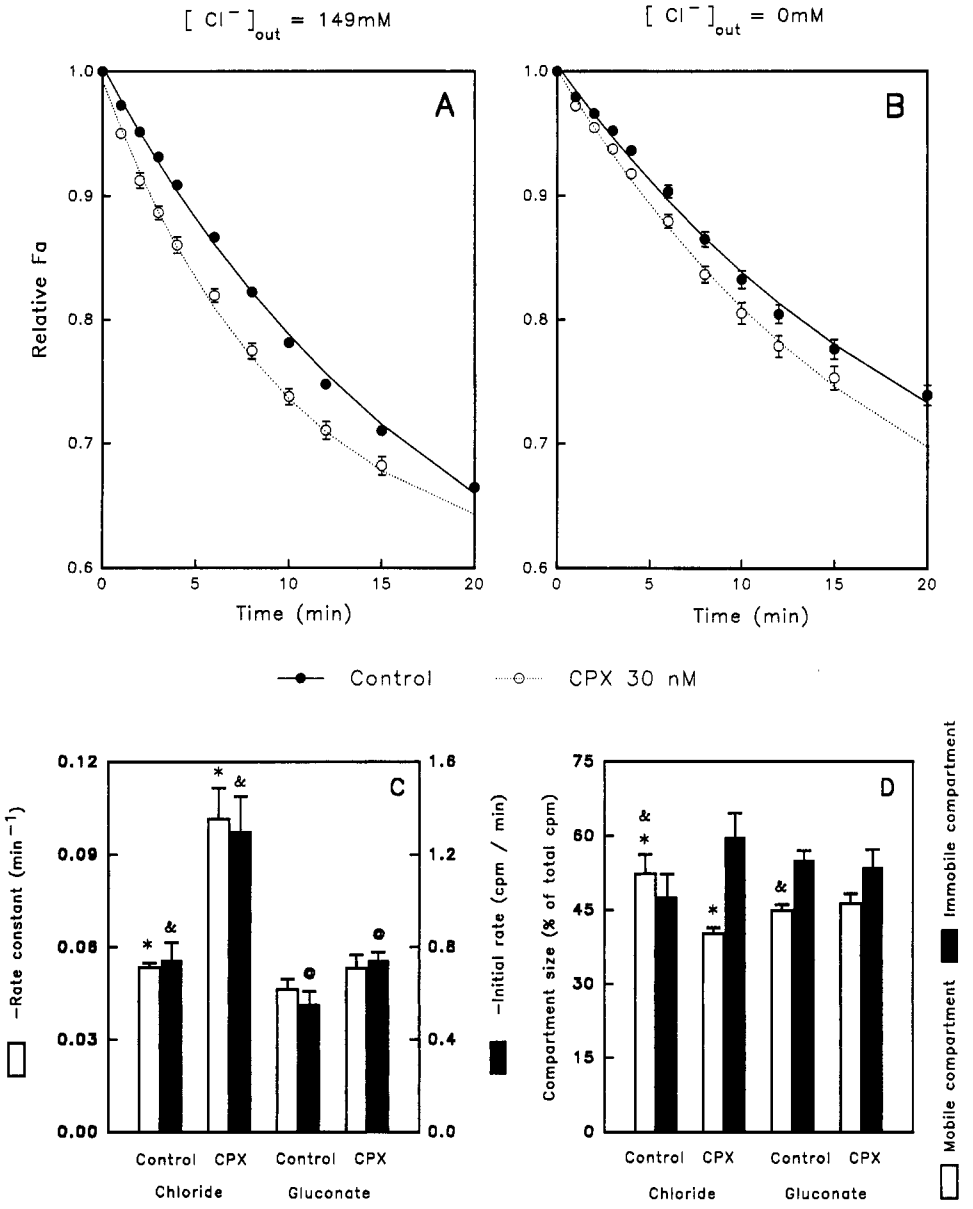

FIGURE 2: Kinetic analysis of ^{36}Cl efflux from CFPAC-1 cells. (A) Efflux of ^{36}Cl from cells into a chloride-containing medium. Control CFPAC-1 cells were equilibrated with ^{36}Cl and allowed to efflux into a medium containing 149 mM chloride (●). A parallel set of cells were treated with 30 nM CPX (○) and the efflux was observed. The lines are the fit of the different data to eq 1. These experiments were performed at 19 °C, in the absence of bicarbonate and in the presence of 100 μM bumetanide. (B) Efflux of ^{36}Cl from cells into a gluconate-containing medium. Cells were treated exactly as in panel A, except that the external medium contained 149 mM sodium gluconate in place of NaCl. (C) Rate constants and initial rates for efflux of ^{36}Cl. The value of the rate constant, k (open bars), was determined from the fit of the data in panels A and B to eq 1 for each of the four conditions. The value of the initial rate (solid bars) was calculated as $v_i = kA$, where k is the rate constant and A is the size of the mobile compartment. The data were normalized to the milligrams of protein in each well. The *, @, and & symbols indicate statistically significant differences between members of each set of data at the $p < 0.05$ level, as determined using the nonpaired Student's t-test. (D) Sizes of the mobile compartment, A (open bars), and the immobile compartment, B (solid bars). The total counts per minute per milligram of protein for each of the cases is as follows: chloride control, 26.5 ± 2.2 (n = 3); chloride + CPX, 31.7 ± 1.6 (n = 3); gluconate control, 26.4 ± 0.8 (n = 3); gluconate + CPX, 30.2 ± 0.7 (n = 3). The * and & symbols indicate statistically significant differences between members of each set of data at the $p < 0.05$ level, as determined using the nonpaired Student's t-test.

By contrast, in gluconate medium the effect of CPX on ^{36}Cl was much less robust. Similar results were observed when the initial rates of the four conditions were compared (see *Figure 2C*). The initial rates were computed from the relationship $v_i = kA$, where k is the rate constant and A is the amplitude in eq 1. The relative sizes of the mobile compartment (A) and immobile compartment (B) are shown in *Figure 2D*. These data thus indicate that the efflux of ^{36}Cl from CFPAC-1 cells occurs from a single compartment, both in the presence and in the absence of CPX activation.

Action of CPX on CFPAC-1, CFPAC-PLJ-CFTR, and CFPAC-PLJ Cells. As shown in *Figure 3A*, CPX activates ^{36}Cl efflux from CFPAC cells in a dose-dependent manner, having a maximal effect at *ca.* 30 nM. The extent of this increase in efflux is 250% of the control level (see *Figure 3B*). Above this concentration, efflux gradually declines toward the control levels. By contrast, CFPAC cells repaired by retrovirus-mediated gene transfer with a full-length wild-type CFTR message (Drumm et al., 1990; CFPAC-PLJ-CFTR) both exhibit a faster apparent control efflux rate and are not further activated by CPX over the entire range of 1−300 nM (see *Figure 3C,D*). Thus these data confirm our previous findings with CPX regarding this cell type (Eidelman et al., 1992). However, in the present set of experiments

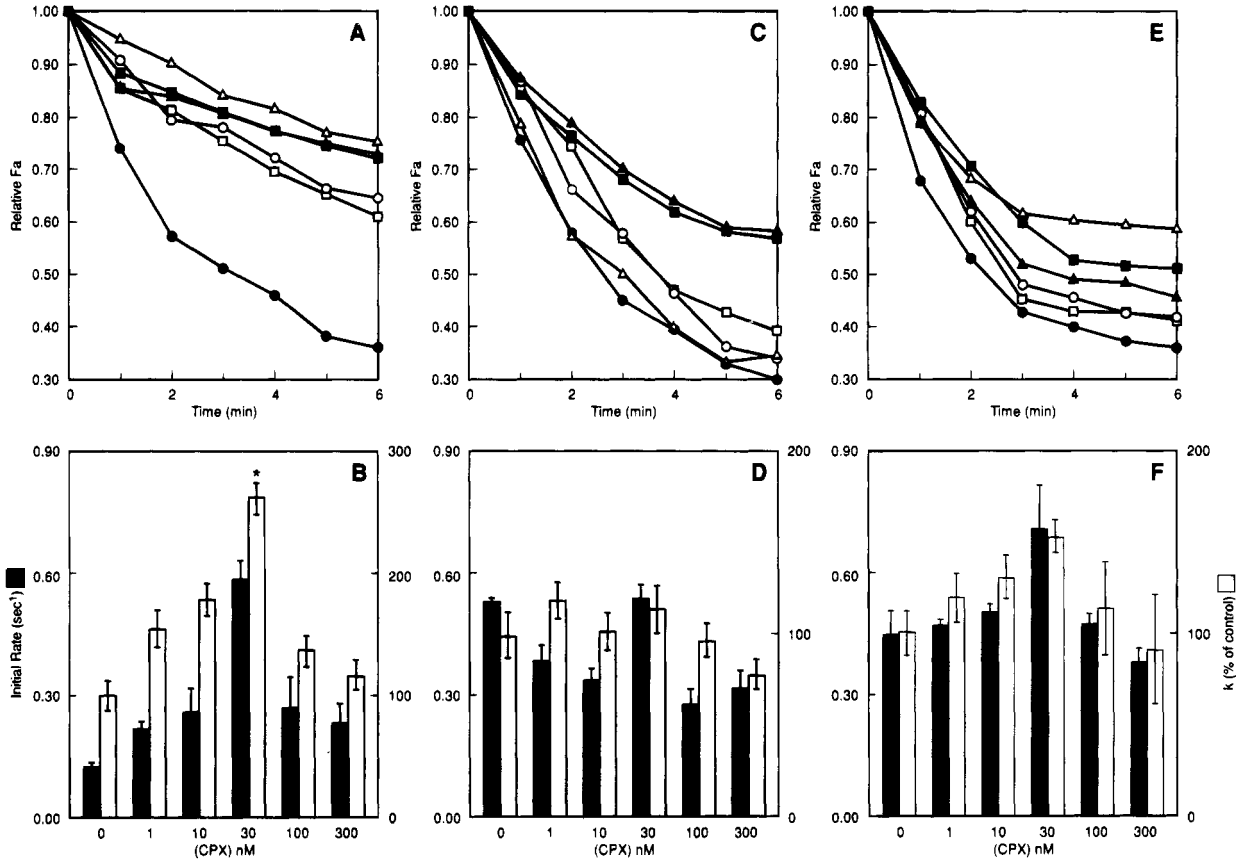

FIGURE 3: Action of CPX on $^{36}Cl^-$ efflux from pancreatic CFPAC-1, CFPAC-PLJ-CFTR, and CFPAC-PLJ cells. Panels A and B are data for CFPAC-1 cells, which naturally express CFTR(ΔF508). Panels C and D are data for CFPAC-PLJ-CFTR, in which the wild-type gene has been transfected into CFPAC-1 cells. Panels E and F are data for CFPAC-PLJ cells, which have been transfected with the retroviral vector alone. Cells were pretreated with the indicated concentrations of CPX for 15 min at 19 °C and quickly washed, and the efflux of $^{36}Cl^-$ was followed for the subsequent 6 min. The concentrations of CPX, maintained throughout the flux experiments, were △ (control), ○ (1 nM CPX), □ (10 nM CPX), ● (30 nM CPX), ▲ (100 nM CPX), and ■ (300 nM CPX). The efflux curves in panels A, C, and E show the fraction of total $^{36}Cl^-$ remaining within the cells (FA) as a function of time. The bar graphs in panels B, D, and F are initial rates (solid bars, left vertical axis) and rate constants (open bars, right vertical axis) of $^{36}Cl^-$ efflux for panels A, C, and E, respectively. These are calculated by linear regression of log (FA) and are presented as a percentage relative to the rate constants of control wells on the same plate. The data points represent the mean ± SEM of four separate, independent experiments ($n = 4$), in which each data point was measured in quadruplicate. The activation of CFPAC-1 cells by 30 nM CPX, relative to either CFPAC-PLJ6 or CFPAC-PLJ4.7, is significant at the $p < 0.001$ level using Student's t-test.

we additionally tested CFPAC-1 cells which had been transfected with the plasmid alone (CFPAC-PLJ). As shown in *Figure 3E,F*, these cells are also activated by CPX over the same concentration range as that found to be effective with the nontransfected CFPAC cells. In addition, as with the CFPAC-1 cells, concentrations of CPX above 30 nM were not as efficacious at stimulating chloride efflux. In sum, the two cell types only differed modestly in the maximum degree of activation by CPX. Thus, the activation of the CFPAC cell by CPX seemed to depend strongly on the *presence* of the CFTR(ΔF508) genotype, as well as on the *absence* of the wild-type CFTR gene. Furthermore, the presence of the carrier plasmid seems to have no influence on the qualitative action of CPX on the CFPAC cells.

Action of CPX on IB3-1 and S9 Cells. We further tested the above hypothesis by examining the effect of CPX on IB3 cells. This cell type is a transformed cell line derived from the tracheal epithelium of a CF patient who underwent a heart–lung transplant. The genotype is ΔF508/W1282X, and there is presently no evidence that the W1282X allele is expressed (Egan et al., 1992). As shown in *Figure 4A*, ^{36}Cl efflux from these cells was activated by CPX in a dose-dependent manner, over virtually the same concentration range that proved effective for the CFPAC cells. In addition, the same maximum extent of activation was noted at 30 nM (*see Figure 4B*), followed by the same type of decline in activity at higher concentrations. By contrast, S9 cells, which are IB3 cells repaired by transfection with the wild-type CFTR sequence (Egan et al., 1992), were virtually unaffected by CPX over the same concentration range (see *Figure 4C,D*). Thus, for both the IB3 and CFPAC cells, the activation of chloride efflux by CPX appears to depend strongly on the *presence* of the CFTR(ΔF508) genotype, as well as on the *absence* of the wild-type CFTR gene.

Action of CPX on 3T3, 3T3(CFTR), and 3T3(CFTR, ΔF508) Cells. However, inasmuch as both the CFPAC and IB3 cells are epithelial cells of human origin, we could not be certain whether the actions of CPX were directed to a process related specifically to the CFTR(ΔF508) molecule or to some other mechanism specific only to (human) epithelial cells. To distinguish between these possibilities, we tested CPX on mouse fibroblast 3T3 cells, permanently transfected with either the wild-type CFTR or the CFTR-(ΔF508) gene. As shown in *Figure 5A*, CPX activated ^{36}Cl efflux from 3T3-CFTR-ΔF508 in a dose-dependent manner. The concentration range for activation appears to be identical to that which had proved effective for both the CFPAC and IB3 cells (see *Figure 5B*). Consistently, CPX treatment of the 3T3-CFTR cells failed to elicit any activation of chloride efflux (see *Figure 5C,D*). Furthermore, a 3T3 cell line,

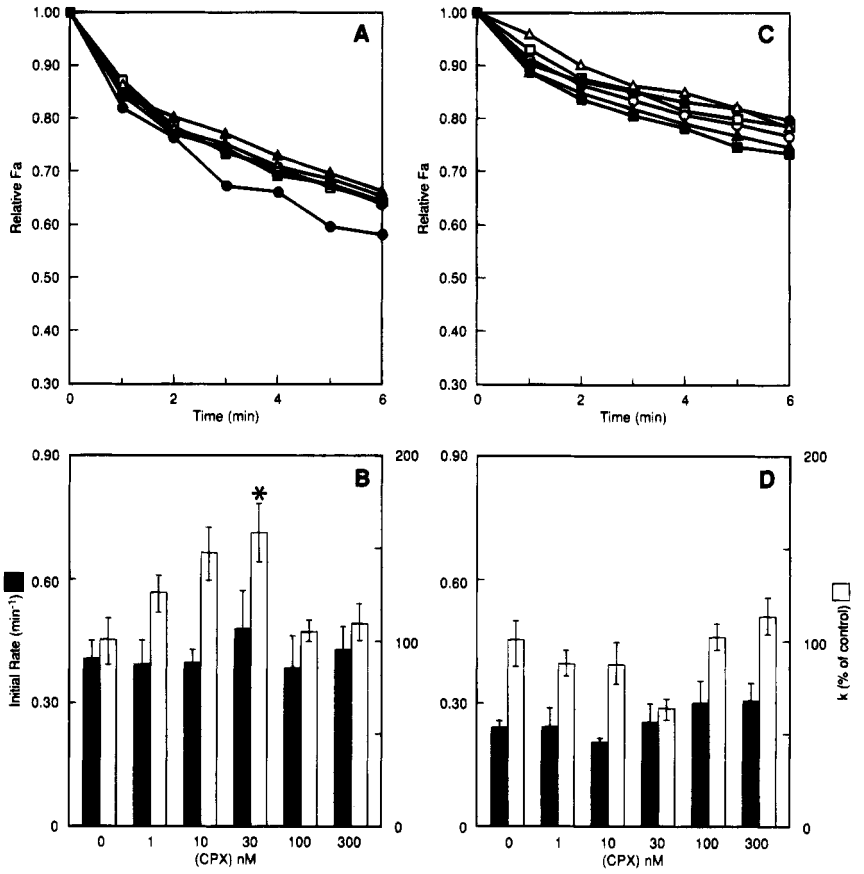

FIGURE 4: Action of CPX on $^{36}Cl^-$ efflux from lung IB3-1 and S9 cells. Panels A and B are data for IB3 cells which naturally express CFTR(ΔF508). Panels C and D are data for S9 cells, which are IB3 cells into which the wild-type CFTR gene has been transfected. Cells were pretreated with the indicated concentrations of CPX for 15 min at 19 °C and quickly washed, and the efflux of $^{36}Cl^-$ was followed for the subsequent 6 min. The concentrations of CPX, maintained throughout the flux experiments, were △ (control), ○ (1 nM CPX), □ (10 nM CPX), ● (30 nM CPX), ▲ (100 nM CPX), and ■ (300 nM CPX). The efflux curves in panels A and C show the fraction of total $^{36}Cl^-$ remaining within the cells (FA) as a function of time. The bar graphs in panels B and D are initial rates (solid bars, left vertical axis) and rate constants (open bars, right vertical axis) of $^{36}Cl^-$ efflux for panels A and C, respectively. These are calculated by linear regression of log (FA) and are presented as a percentage relative to the rate constants of control wells on the same plate. The data points represent the mean ± SEM of four separate, independent experiments (n = 4). The action of 30 nM CPX on IB3 cells, relative to S9 cells, is significant at the $p < 0.001$ level using Student's t-test.

selected at random from the American Type Culture Collection (ATCC), proved marginally sensitive (*Figure 5E,F*). We term the effect marginal because if one compares the *rate constant* (open bars) for 30 nM CPX with the control, it is statistically not different from that of the 0 nM CPX condition. However, the rate constant (open bars) and initial rate (solid bars) for 1 nM CPX are greater than all other equivalent values shown in *Figure 5F*, including that for 0 nM CPX. Nonetheless, there was no systematic titration, although, since each data point is calculated from a total of 64 points, we must presume that each is accurate. We conclude from this result that CPX activation appears to be directed to a process related specifically to the expression of the CFTR(ΔF508) molecule and not necessarily to a separate mechanism specific only to (human) epithelial cells, nor to any mechanism related to the transfection process.

Action of Different A_1 Antagonists on Chloride Efflux from CFPAC Cells. In anticipation of possibly finding a drug more efficacious than CPX, we assessed the action of other A_1 receptor antagonists on ^{36}Cl efflux from the CF cells IB3 and CFPAC. We planned to devote further attention to a compound only if it acted on *both* of our CF cell types. We tested six different high-affinity A_1 receptor antagonists, whose structures are shown in Figure 6. These antagonists are given here, with the values of the apparent K_i for displacement of an A_1 agonist such as [^{3}H]-N^6-(phenylisopropyl)adenosine (PIA) in rat brain membranes (Ukena et al., 1986) given parenthetically. These compounds included 2-thio-CPX (Shimada et al., 1992; K_i = 1 nM), KW-3902 (8-noradamantyl-1,3-dipropylxanthine) (Jacobson et al., 1988; K_i = 1.3 nM), CPT (8-cyclopentyl-1,3-dimethylxanthine) (Shimada et al., 1992; K_i = 10 nM), 3,4-dehydro-CPX (Ukena et al., 1986; K_i = 45 nM), 3-F-CPX (Ukena et al., 1986; K_i = 42 nM), 3-I-CPX (Ukena et al., 1986; K_i = 58 nM). In the same system, the K_i of CPX is *ca.* 0.9 nM. However, as shown in Figure 7, we found that each of these compounds had only a marginal effect or no effect whatsoever on ^{36}Cl efflux from CFPAC cells in the concentration range of 1−300 nM. Two compounds, 3-F-CPX and KW-3902, seemed to activate chloride efflux slightly in some experiments but not all. We have therefore focused our attention on CPX and excluded these other compounds from further scrutiny.

Detection of CFTR Antigen in Cell Lines. The effect of CPX on chloride efflux is evident in cells expressing CFTR-(ΔF508) but appeared lost when the cells were repaired by transfection with wild-type CFTR. To verify that CFTR was in fact being expressed in repaired cells, we examined the different cells by Western blot analysis. As shown in *Figure 8* (lanes 2 and 6), 3T3-CFTR cells and CFPAC-CFTR cells, respectively, expressed CFTR at substantial levels. As a control, we measured CFTR levels in HT29 colon carcinoma

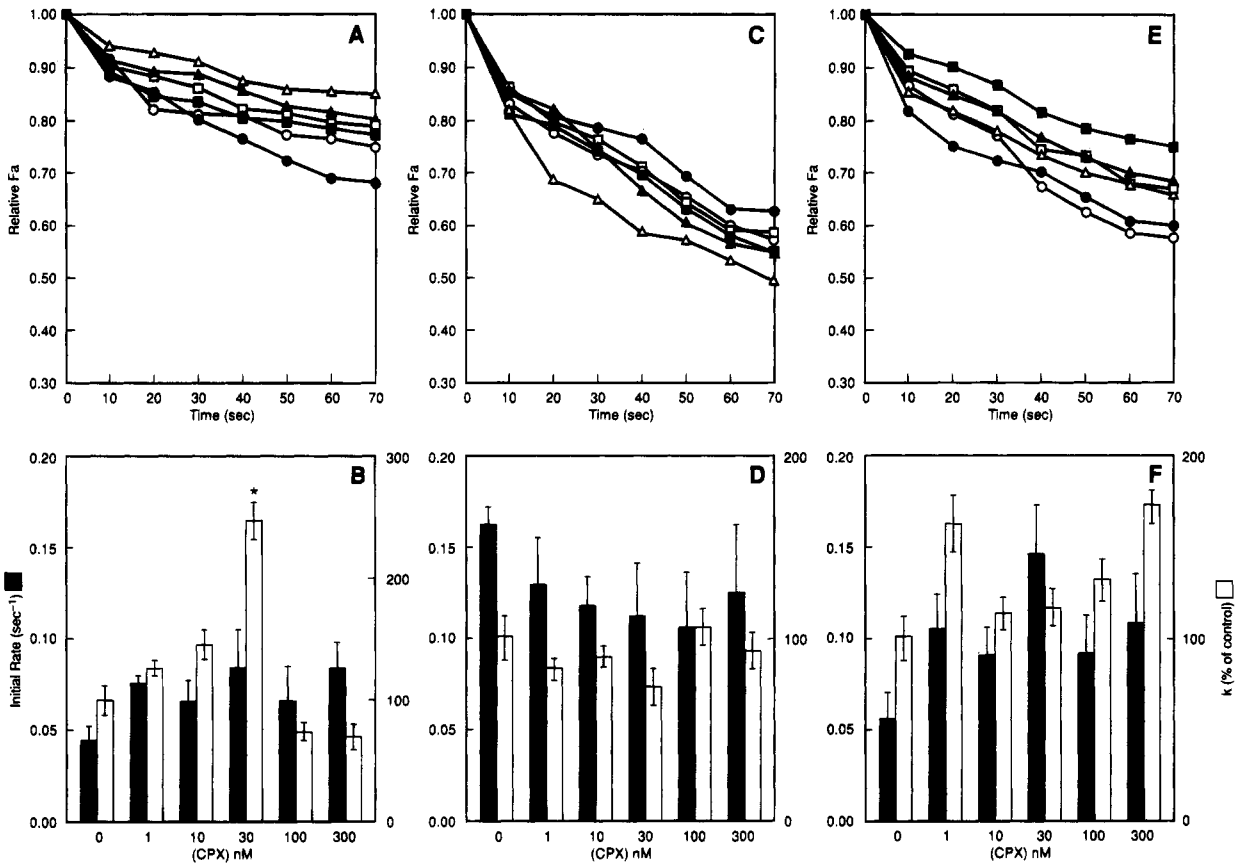

FIGURE 5: Action of CPX on $^{36}Cl^-$ efflux from mouse NIH-3T3 (CFTR), NIH-3T3 (CFTR,ΔF508), and control NIH-3T3 fibroblasts. Panels A and B are data for NIH-3T3 cells which have been transfected with CFTR(ΔF508). Panels C and D are data for NIH-3T3 cells into which the wild-type CFTR gene has been transfected. Panels E and F are data for control NIH-3T3 cells. The bar graphs in panels B, D, and F are initial rates (solid bars, left vertical axis) and rate constants (open bars, right vertical axis) of $^{36}Cl^-$ efflux for panels A, C, and E, respectively. Cells were pretreated with the indicated concentrations of CPX for 15 min at 19 °C and quickly washed, and the efflux of $^{36}Cl^-$ was followed for the subsequent 70 s. The concentrations of CPX, maintained throughout the flux experiments, were △ (control), ○ (1 nM CPX), □ (10 nM CPX), ● (30 nM CPX), ▲ (100 nM CPX), and ■ (300 nM CPX). Data were calculated exactly as for experiments in Figures 1 and 2. The data points represent the mean ± SEM of four separate, independent experiments ($n = 4$). The action of 30 nM CPX on 3T3-CFTR ΔF508 cells, relative to either control 3T3 cells or 3T3-CFTR cells, is significant at the $p < 0.001$ level using Student's t-test.

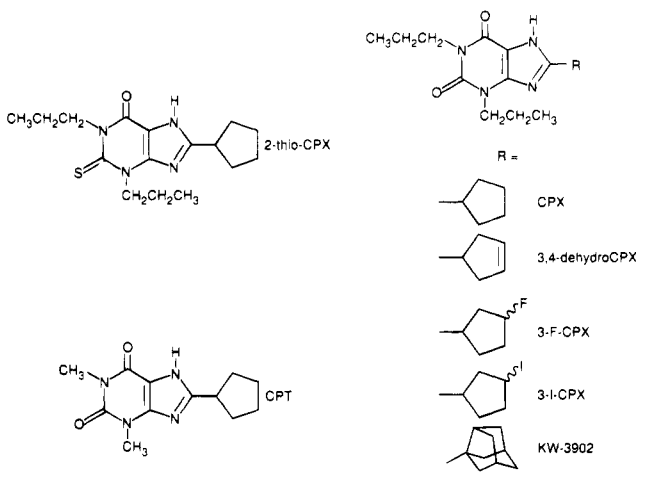

FIGURE 6: Structures of A_1 receptor antagonists having homology to CPX. The structure in the upper right-hand corner is the common structure, and R is any of the groups shown below along the right-hand margin. On the upper left-hand side of the figure is 2-thio-CPX, in which the carbonyl at the 2-position is replaced by sulfur. On the lower left-hand side of the figure is CPT (cyclopentyltheophylline), in which the propyl groups on CPX are replaced by methyl groups.

cells, either untreated (*Figure 8*, lane 9) or treated overnight with 10 μM forskolin to raise the expression of CFTR (Breuer et al., 1992) (*Figure 8*, lane 10). In some cell lines, including the 3T3 cells from the ATCC, we detected additional minor or lower molecular weight immunoreactive bands, but in this paper we avoid any explicit attention to their identity. By contrast, the levels of expression of transfected CFTR(ΔF508) in the 3T3 cells were too low for detection by our method (*Figure 8*, lane 3). In addition, we could not detect endogenous CFTR(ΔF508) in CFPAC-1 cells (*Figure 8*, lanes 4 and 5). Similarly, our method was not sufficiently sensitive to detect CFTR(ΔF508) or CFTR expression in IB3-1 (*Figure 8*, lane 7) and S9 cells (*Figure 8*, lane 8), respectively. However, Egan et al. (1992) have detected increased levels of CFTR in repaired S9 cells using different antibodies and methods.

DISCUSSION

We conclude from these studies that CPX can activate chloride efflux from CF epithelial cells such as CFPAC and IB3 if the CFTR(ΔF508) genotype is *present* and the wild-type CFTR gene is *absent*. In the NIH 3T3 cells, one can only conclude that the CPX effect depends on having the mutant CFTR(ΔF508) present, since the CPX effect is not discernible in the recombinant 3T3 cells expressing wild-type CFTR. Furthermore, it appears that the nature of the

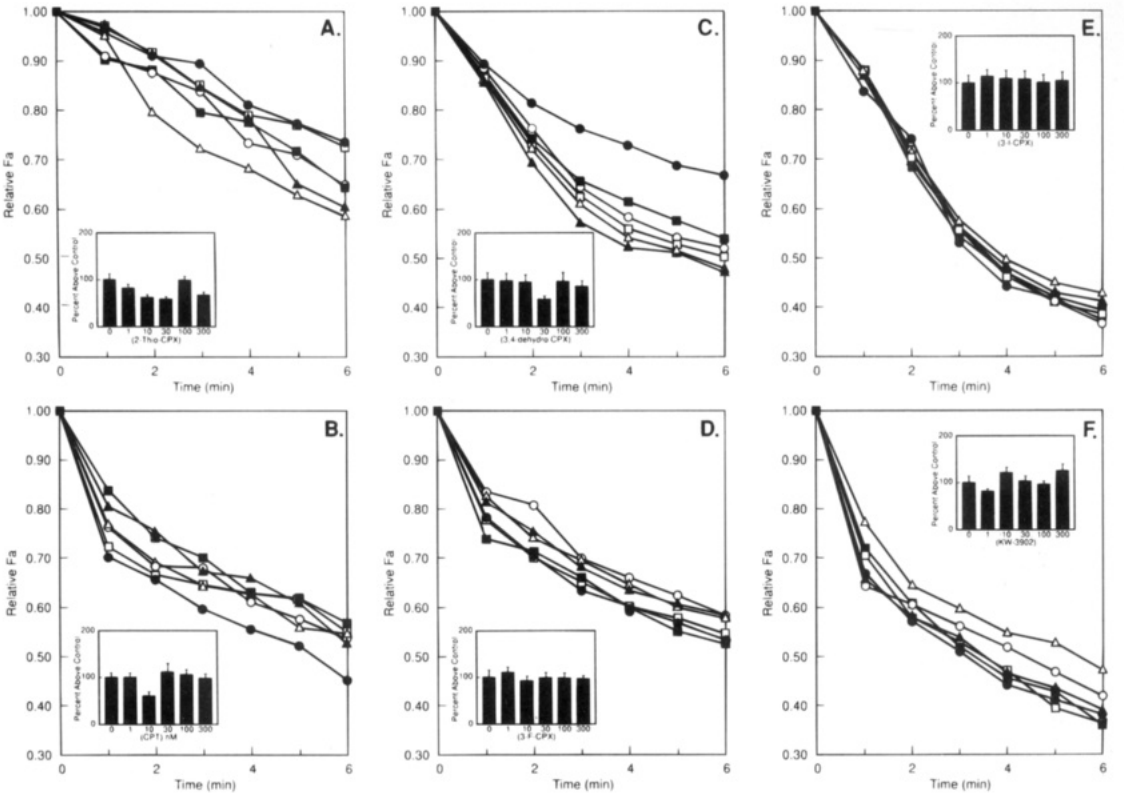

FIGURE 7: Effects of different A_1 receptor antagonists on $^{36}Cl^-$ efflux. Six A_1 receptor antagonists, having homology to CPX, have no effect on $^{36}Cl^-$ efflux from pancreatic CFPAC-1 cells. CFPAC-1 cells naturally bear the CFTR(ΔF508) mutation. Cells were treated exactly as described in the legend to Figure 3, except that CPX was replaced by (A) 2-thio-CPX, (B) CPT, (C) 3,4-dehydro-CPX, (D) 3-F-CPX, (E) 3-I-CPX, or (F) KW-3902. The concentrations of different drugs were △ (control), ○ (1 nM), □ (10 nM), ● (30 nM), ▲ (100 nM), and ■ (300 nM). The individual inset bar graphs show the relative efflux constants, evaluated exactly as described for CPX in the legend to Figure 3.

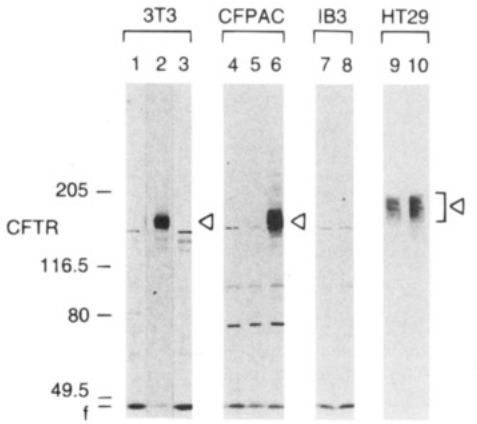

FIGURE 8: Expression of CFTR immunoreactivity in cell lines used for analysis of ^{36}Cl fluxes. The presence of CFTR in normal and transfected cells was detected by Western blotting as described in Materials and Methods, using the monoclonal anti-CFTR antibody M3A7. In order to enhance detection by the ECL method, low-level expression was detected after overnight (ON) exposure, while shorter times of exposure, indicated parenthetically, were employed for higher levels of expression. Lane 1, wild-type 3T3 cells (ON); lane 2, CFTR-transfected 3T3 cells (18 min); lane 3, CFTR-ΔF508-transfected 3T3 cells (ON); lane 4, CFPAC cells (ON); lane 5, CFPAC-PLJ-6 (ON); lane 6, CFPAC-PLJ4.7 (ON); lane 7, IB3 cells (ON); lane 8, S9 cells (ON); lane 9, HT29 cells (5 min); lane 10, HT29 cells, after overnight treatment with 10 μM forskolin (5 min). Open arrowhead delineates position of CFTR.

vector system carrying the wild-type CFTR into the target cell has no impact, *per se*, on the loss of the CPX effect. In the case of the pancreatic CFPAC cell, the vector system is a retroviral system (Drumm et al., 1990), while for the IB3 cell, the vector system is an adeno-associated virus system (Egan et al., 1992). In the recombinant 3T3 cells, the CFTR and the CFTR(ΔF508) genes were carried by a different system (Anderson et al., 1991b).

These data also show unambiguously that while CPX (Eidelman et al., 1992) is able to active the chloride efflux process, six other well-characterized A_1 antagonists were not able to do so. The mechanism by which CPX acts on cells carrying the CFTR(ΔF508) genotype now merits further scrutiny in the light of a substantial amount of pharmacological and biochemical data presently available on A_1 receptors. The affinity of CPX for A_1 receptors can vary substantially across phylogeny, especially when tissues are compared in a cell-free binding assay (Ukena et al., 1986). However, cellular constituents such as GTP (Martens et al., 1988) and extracellular constituents such as magnesium (Parkinson & Fredholm, 1992) can change the binding properties of A_1 receptors. For these reasons, the apparently similar affinities of CPX for both the human and mouse cells need not immediately direct our attention away from classical A_1 receptors, *per se*, as the hypothetical common sites of CPX action.

At present, there is no experience with A_1 receptors being selectively sensitive to CPX and XAC but not to other closely related analogues. If, in fact, the activation mechanism involved classical A_1 receptors, then the analogues described in Figure 6 should all have been active at some concentration. The structural modifications to CPX were not profound and included CPX substituted at the 3-position on the cyclopentyl ring with either fluorine or iodine and CPX with a double bond replacing the single bond between carbons 3 and 4 on

the cyclopentyl ring. Yet they are all inactive. Furthermore, both KW-3902 and 2-thio-CPX, possessing a noradamantyl group in place of the cyclopentyl ring or a thiocarbonyl group at the purine C2 position, respectively, have nearly identical affinities to that for CPX on rat brain membranes. Yet they are also inactive. These data, with a large number of different, well-characterized, and selective A_1 antagonists, must therefore lead us to question the hypothesis that the CPX effects on CF cells occur via interactions with a classical A_1 receptor.

This concern is magnified by the observation that mouse 3T3 cells, transfected with either CFTR or CFTR(ΔF508), differentially respond to CPX just as do the human epithelial CF cells. Alternative explanations for CPX action could include either action of CPX on a function of CFTR(ΔF508) molecules *per se*, action of CPX on a chloride channel common to these cells, or action of CPX on a type of purine receptor common to these different cells that has not hitherto been described. With respect to the possibility that CPX might interact with CFTR, Sorscher and colleagues have reported that the nonselective adenosine receptor antagonist 3-isobutyl-1-methylxanthine (IBMX) binds to the recombinant wild-type or ΔF508 forms of the first nucleotide binding fold (NBF-1) of CFTR and displaces the ATP analogue TNP-ATP (Logan et al., 1992). The apparent K_i value for IBMX is *ca.* 2 mM, a concentration reported to be useful to activate a chloride current associated with recombinant CFTR-(ΔF508) expressed in *Xenopus* oocytes (Drumm et al., 1991). However, this IBMX concentration is substantially greater than the 40 μM level we used to help activate chloride efflux from CFPAC-1 cells (Eidelman et al., 1992). More recently, it has been reported that xanthines such as IBMX and theophylline, at 1 mM concentrations, are able to retard CFTR channel rundown under conditions where ATP is absent from the system (Becq et al., 1993). Furthermore, in the absence of ATP these same compounds increase the open-channel probability of wild-type CFTR (Becq et al., 1993). Finally, we cannot exclude the possibility that CPX acts on a different site which is nonetheless common to these different cells. For example, it has recently been shown that a wide variety of nonepithelial cells, used extensively to study recombinant CFTR functions, express a small, cAMP-independent, ohmic anion channel similar to that hitherto associated with CFTR activity (Gabriel et al., 1992). However, it is very unlikely that CPX accesses this latter channel since CPX is inactive in control 3T3 cells.

We conclude that whatever the mechanism by which CPX activates chloride efflux, it is similarly manifest on cells expressing the CFTR(ΔF508) mutation. On the basis of previous results from studies on CFPAC-1 cells and a survey of the literature indicating the low intrinsic toxicity of CPX in different organisms, we have suggested CPX as a candidate drug to treat cystic fibrosis (Eidelman et al., 1992). The present data lend further support this suggestion and further indicate that the class of receptor molecules which interact with high affinity and selective purinergic compounds may be broader than hitherto considered. In the present case, the structural requirements for the action of CPX on cells expressing the CFTR(ΔF508) mutation are more narrowly defined than is the action of CPX at classical A_1 receptors.

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