Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5562–5566, June 1992
Medical Sciences

A_1 adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells

(CFPAC cells/xanthines)

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Communicated by Bernard Witkop, February 10, 1992 (received for review December 6, 1991)

ABSTRACT **A_1 adenosine-receptor-antagonist drugs such as 8-cyclopentyl-1,3-dipropylxanthine (CPX) and xanthine amine congener (XAC) are found to activate the efflux of $^{36}Cl^-$ from CFPAC cells. These cells are a pancreatic adenocarcinoma cell line derived from a cystic fibrosis (CF) patient homozygous for the common mutation, deletion of Phe-508. The active concentrations for these compounds are in the low nanomolar range, consistent with action on A_1 adenosine receptors. In addition, drug action can be blocked by exogenous agonists such as 2-chloroadenosine and also can be antagonized by removal of endogenous agonists by treatment with adenosine deaminase. Cells lacking the CF genotype and phenotype, such as HT-29 and T84 colon carcinoma cell lines, appear to be resistant to activation of chloride efflux by either drug. CFPAC cells transfected with the CF transmembrane regulator gene, *CFTR*, are also resistant to activation by CPX. We conclude that, since these antagonists are of relatively low toxicity and appear to act somewhat selectively, they might be considered as promising therapeutic candidates for CF.**

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene (1–3), which result in an abnormal potential difference across CF epithelia (4–10). This abnormality is due to a reduced apical Cl^- conductance (11–13), which is apparently responsible for consequent pathophysiological changes in epithelial tissues of lung, pancreas, and related tissues in the gastrointestinal tract and elsewhere (14). Indeed, the presence of mRNA for CFTR in cultured epithelial cells has been shown to be closely correlated with cAMP-dependent $^{36}Cl^-$ efflux activity (15). Repair of this anion conductance deficit in patients has been the goal of gene therapy and has been achieved in cultures of CFPAC cells supplemented with recombinant wild-type CFTR (16). CFPAC cells are pancreatic adenocarcinoma cells from a CF patient homozygous for the most common mutation, deletion of Phe-508 (17). We have chosen the complementary therapeutic approach of searching for drugs that could either activate the resident mutant *CFTR* gene product or cause additional secretion of Cl^- from the affected cell. Indeed, encouraging clinical results have been reported recently using aerosols of amiloride (18) or a mixture of ATP and UTP (19), which retard Cl^- accumulation by tracheal epithelium. In our present report we describe activation of Cl^- efflux from CFPAC cells by drugs from the class of A_1 adenosine-receptor antagonists. One of these drugs, 8-cyclopentyl-1,3-dipropylxanthine (CPX), is particularly promising from the viewpoint of potential therapeutic application, since it is a potent low-toxicity drug that specifically activates Cl^- efflux from CF cells.

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MATERIALS AND METHODS

Culture of CFPAC Cells. CFPAC cells and *CFTR*-transfected CFPAC cells (CFPAC-4.7 CFTR) were obtained from R. Frizzell, University of Alabama at Birmingham, Birmingham, AL. The cells were split and seeded at low density on 24-well Costar plates in medium composed of Eagle's minimal essential medium with Dulbecco's modifications (DMEM), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin at 300 units/ml, streptomycin at 300 μg/ml, and 1% (wt/vol) glutamine. After 5 hr the medium was replaced, and attached cells were allowed to reach confluency during a period of 48 hr at 37°C in 5% CO_2/95% air.

Measurement of Cl^- Efflux. Before each experiment, cells were loaded with $^{36}Cl^-$ as follows. Confluent cells were washed four times in DMEM (bicarbonate free). Then, after aspirating the last wash, 250 μl of DMEM was added to each well and supplemented with 25 μl (*ca.* 1.4×10^8 cpm) of $^{36}Cl^-$ (Amersham). Plates of cells were then incubated at 37°C for 2 hr in a CO_2-free incubator. Plates were then moved to 25°C, and drugs were added at the concentrations and for the times shown below. After the incubation period, cells were washed four times in 500 μl of an ice-cold wash medium composed of 150 mM sodium gluconate and 10 mM Hepes (pH 7.4). At the end of the wash step, 500 μl of flux medium at 21°C was added, and sampling was initiated by collecting 50-μl aliquots from each well at 0, 1, 2, 3, 5, 7, and 10 min. The flux medium consisted of 150 mM sodium gluconate, 1.5 mM potassium gluconate, 10 mM sodium Hepes (pH 7.4), 100 μM bumetanide to inhibit the cotransporter, and different drugs as required. The osmolarity was 310 mOsm. At the end of each flux experiment, 20 μl of 50% trichloroacetic acid was added to a final concentration of 5% to obtain a measure of remaining radioactivity. Samples were mixed with 1.5 ml of Cytoscint fluid and assayed for 2 min on a Beckman LS9000 scintillation counter with windows at maximum width.

Drug Treatment of Cells. Cells were pretreated with drugs (RBI, Natick, MA) as follows: forskolin (4 μM for 5 min), isobutylmethylxanthine (IBMX; 40 μM for 5 min), xanthine amino congener (XAC) or CPX (various concentrations for 15 min), 2-chloroadenosine (100 μM for 30 min), and adenosine deaminase (2 units/ml for 30 min). Data from the scintillation counter were transferred by Data Module to a computer for analysis.

Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane regulator; CPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener; IBMX, isobutylmethylxanthine.

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RESULTS

Cl^- Efflux from CFPAC Cells. In initial studies of $^{36}Cl^-$ efflux from CFPAC cells, we noted that exposure of the cells to a mixture of forskolin and IBMX or to IBMX alone resulted in a modestly enhanced rate of Cl^- efflux (Fig. 1). Efflux from untreated CFPAC cells was only modestly affected by exposure to either IBMX (40 μM) or forskolin (4 μM) but was substantially enhanced in the presence of both drugs (Fig. 1). These data were fit to first-order curves, and we found that while IBMX and forskolin individually had some effect on the rate of efflux, the combination of the two drugs caused an increase in the initial efflux rate of *ca.* 75% over the control rate. Initial rates were statistically different from control.

However, in these and other experiments to be described, we were concerned about the possibility of multiple components to the efflux processes; therefore, we directed particular attention to the error analysis in the fit of the curves to the first-order equations. Furthermore, in different experiments we noted that the baseline (control) levels of $^{36}Cl^-$ efflux varied to some degree. Therefore, in the experiments reported here we compared all results to controls run at the same time under otherwise identical conditions.

A_1 Adenosine-Receptor Antagonists Activate Cl^- Efflux from CFPAC Cells. The action of IBMX is known to include not only inhibition of phosphodiesterase (20) but also antagonist activity on adenosine receptors (21). Additional experiments with the selective phosphodiesterase inhibitor rolipram (22) failed to show an effect on Cl^- fluxes in CFPAC cells; therefore, our attention turned to high-affinity adenosine receptor antagonists (23). Several of these drugs are depicted in Fig. 2.

Of an initial set of adenosine antagonists tested for ability to evoke Cl^- efflux from CFPAC cells, we found that XAC was somewhat effective at low concentrations (Fig. 3*A*). Relative rate constants for the efflux evoked by XAC at two concentrations compared with control are summarized in Fig. 3*B*. From these data it is apparent that XAC at a concentration of only 10 nM was able to increase the rate of $^{36}Cl^-$ efflux from CFPAC cells by *ca.* 50%. Furthermore, at higher concentrations of XAC (e.g., $\geq$1 μM), the chloride efflux rate returned toward the control rate. This result was consistent with the concept that the action of lower concentrations of XAC might be upon A_1 adenosine receptors (23) and that the loss of activity at higher concentrations might reflect either nonspecific actions or actions on A_2 adenosine receptors.

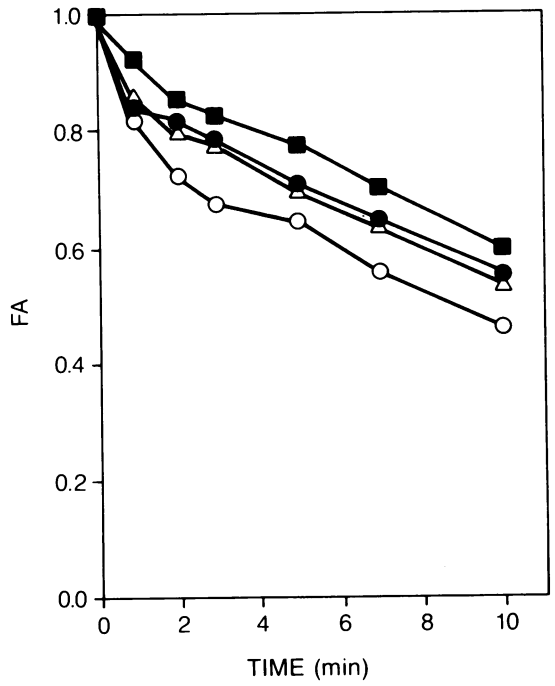

FIG. 1. Cl^- efflux from CFPAC cells. The vertical axis represents the fraction of total $^{36}Cl^-$ remaining in the cell (FA) at the given time point. Cells were treated with IBMX (●), forskolin (△), or forskolin + IBMX (○) or were controls (■).

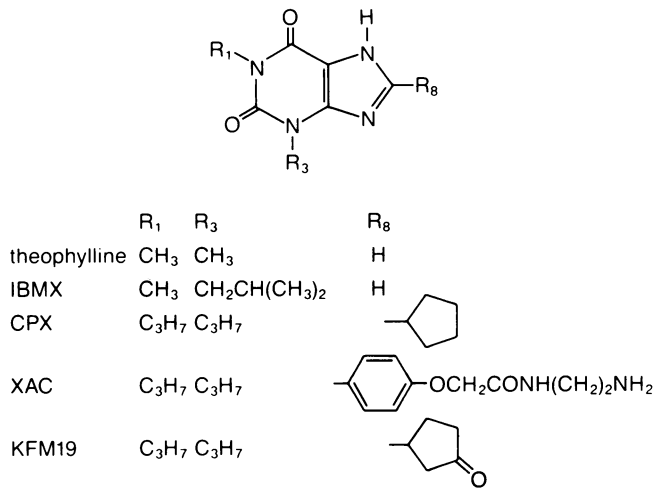

FIG. 2. Xanthine-analogue adenosine-receptor-antagonist drugs. Theophylline and IBMX are nonselective for A_1 and A_2 adenosine receptors. In rats, XAC and CPX are highly selective for A_1 receptors. At higher concentrations, they both block A_2 receptors. In humans, CPX is *ca.* 50-fold selective for A_1 receptors (24). KFM19 is A_1 selective (25).

As a further test of this hypothesis, we also examined the actions of CPX (see Fig. 2), which is known to be somewhat more potent and more selective than XAC for human A_1 adenosine receptors (26–28). CPX also caused profound activation of $^{36}Cl^-$ efflux at a concentration of 10 nM (Fig. 4). Furthermore, as anticipated from the data with XAC, CPX became less effective at higher concentrations. Eventually CPX, at a concentration of 10 μM, reduced the rate of $^{36}Cl^-$ efflux to a value below that of the control rate. In contrast with these results with CFPAC cells, tests of XAC or CPX on HT-29 or T84 cells or of CPX on *CFTR*-transfected CFPAC cells failed to evoke changes in Cl^- efflux (data not shown).

Control by Adenosine Agonist and Adenosine Deaminase. As yet a further test of the hypothesis that adenosine receptors could be utilized to enhance Cl^- efflux from CFPAC cells, we examined the relative effects of 2-chloroadenosine, a potent adenosine agonist (28), and adenosine deaminase on XAC activation of efflux. We anticipated that if endogenous adenosine were responsible for the tonic A_1 agonist activity upon which XAC or CPX could act, then antagonist drug efficacy should be reduced by the action of adenosine deaminase on endogenous adenosine. Similarly, the action of 2-chloroadenosine should be to suppress Cl^- efflux below that already suppressed by endogenous adenosine and to possibly interfere with the action of XAC or CPX. Fig. 5*A* shows that adenosine deaminase permitted only modest increases in the efflux rate upon the further addition of XAC. Relative efflux rate constants in Fig. 5*B* show that the suppression of the efflux rate at higher XAC concentration is still evident. Finally, 10 μM 2-chloroadenosine proved to be a potent inhibitor of XAC action on Cl^- efflux from CFPAC cells. The action of 2-chloroadenosine (Fig. 5) was to blunt the XAC effect on Cl^- efflux. However, the highest dose level of XAC (300 μM) still reduced efflux rates to values below control levels (not shown).

DISCUSSION

We conclude from these studies that it is possible to activate Cl^- efflux from CFPAC cells by using a selective A_1 adeno-

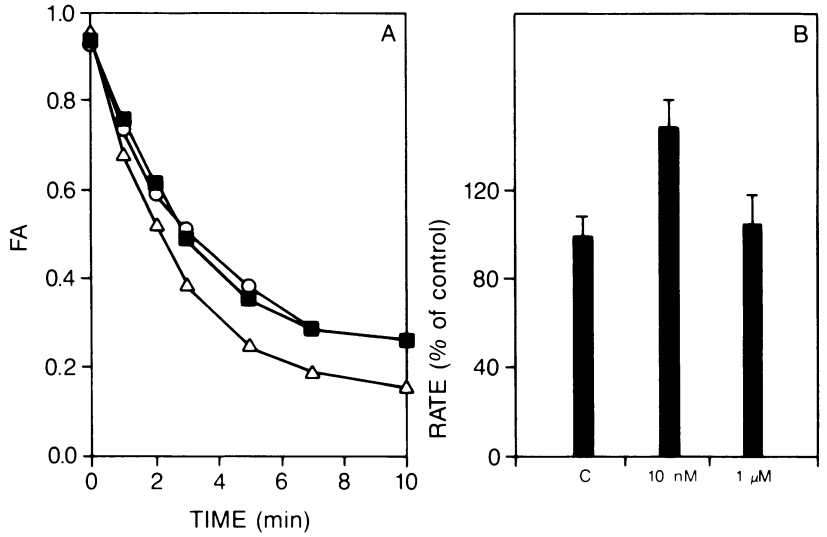

FIG. 3. Influence of XAC on $^{36}Cl^-$ efflux from CFPAC cells. (*A*) The fraction of $^{36}Cl^-$ remaining in the cells (FA) is plotted against time during the efflux process in a typical experiment. ■, Control; △, 10 nM XAC; ○, 1 μM XAC. (*B*) The relative rate constants of $^{36}Cl^-$ efflux is shown for zero, 10 nM, and 1 μM XAC. The rate was calculated from a curve fit to a first-order exponential function. The error bars show the SEM for four experiments.

sine-receptor antagonist such as CPX and that such an approach may constitute a therapeutic modality with clinical value in the near term. The apparent specificity of this reaction for CF cells is illustrated by our experience that neither XAC nor CPX has discernible effects on Cl^- efflux from the colon carcinoma cell lines HT-29 and T84. Both of these cell lines possess the wild-type CFTR message. Furthermore, CPX had no effect on *CFTR*-transfected CFPAC cells. This observation could indicate that in these control cells, the Cl^- efflux process is fully active and cannot be further activated. Alternatively, a putative second pathway of Cl^- efflux, identified in CFPAC cells, might be either absent or inactive in these cells. In either of these cases, the potential therapeutic advantages of a drug able to activate Cl^- efflux from CF cells specifically would be appreciable, since drug action would tend to be less encumbered by unwanted side effects from unaffected tissues. Preliminary studies also show that CPX can activate Cl^- efflux from IB3 cells, a simian virus 40-transformed CF tracheal epithelial cell line carrying the Phe-508 deletion and an unknown *CFTR* mutation (W. B. Guggino, P. L. Zeitlin, and all authors, unpublished data).

We now can direct our attention to known mechanisms regulating Cl^- transport in control cells and ask whether CPX action might be mediated by any of these processes. Cl^- permeability in the gastrointestinal tract is known to be activated by adenosine action on A_2 receptors and subsequent activation of adenylate cyclase. This has been shown for both mammalian ileum (29) and the human colon carcinoma cell line T84 (30). Presumably, CF cells cannot be activated by adenosine binding to putative A_2 receptors

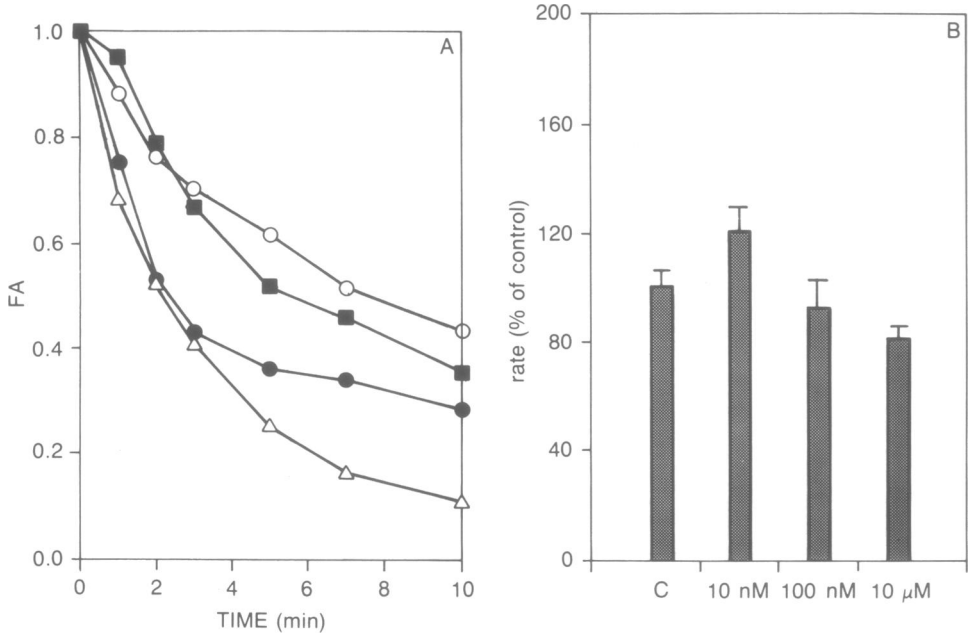

FIG. 4. Influence of CPX on $^{36}Cl^-$ efflux from CFPAC cells. (*A*) The fraction of $^{36}Cl^-$ remaining in the cells (FA) is plotted against time during the efflux process. ■, Control; △, 10 nM CPX; ●, 100 nM CPX; ○, 10 μM CPX. This experiment is representative of four experiments. (*B*) Rate of Cl^- efflux is shown in the presence of the different concentrations of CPX in *A*. The rate constants were evaluated from a fit of the efflux data to a first-order rate equation. The error bars shown are those for the calculation of the efflux rate constants in *A*.

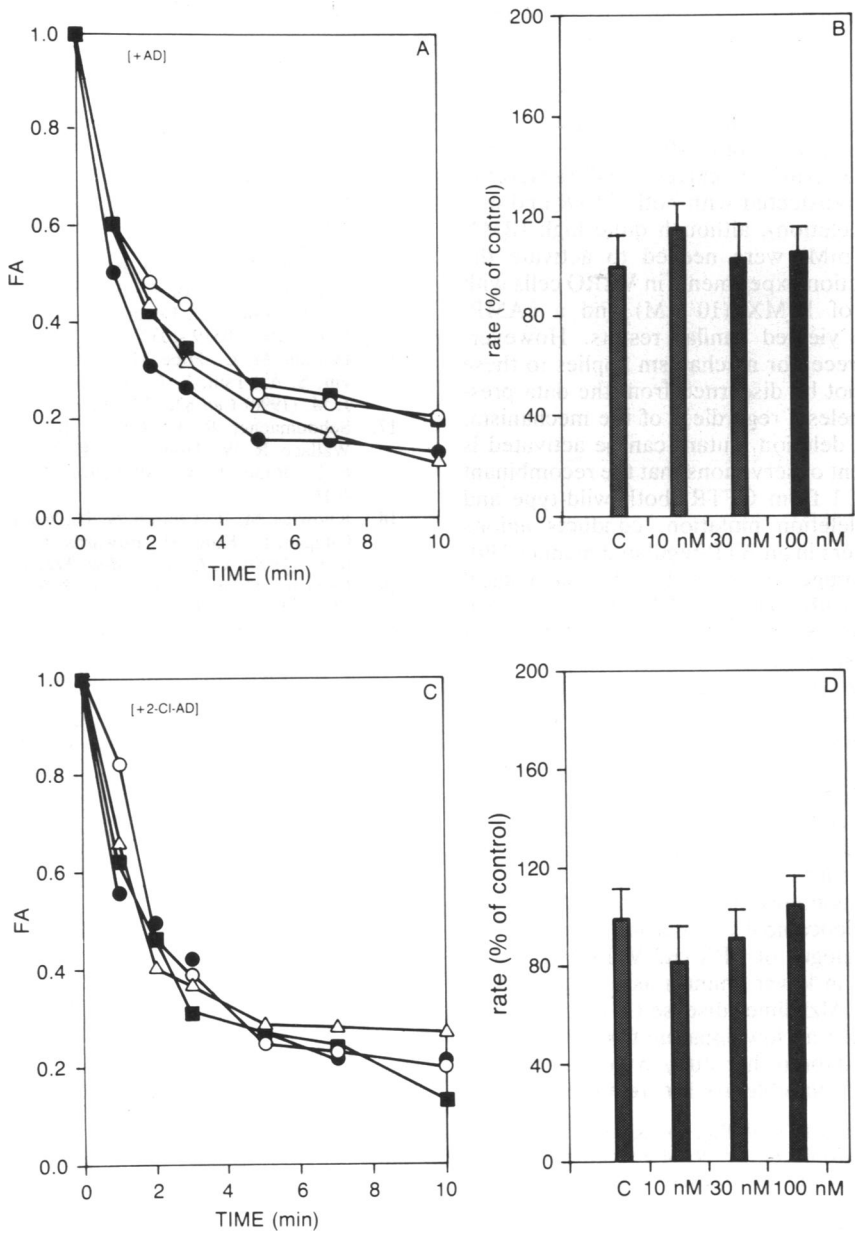

FIG. 5. Influence of adenosine deaminase and 2-cloroadenosine on the response of CFPAC cells to XAC. (*A*) Cells were pretreated with adenosine deaminase and then XAC as described in the legend to Fig. 1, and the $^{36}Cl^-$ efflux was measured. ■, Control; △, 10 nM XAC; ●, 30 nM XAC; ○, 100 nM XAC. (*B*) The relative rate constants of efflux compared with the control (*C*) rate are shown in the bar graph. The rate constants were calculated from the fit of the efflux data to a first-order rate equation. (*C*) Influence of 2-chloroadenosine on $^{36}Cl^-$ efflux is shown. The symbols are exactly as shown in *A*. (*D*) Relative rate constants of efflux for the data in *C* as compared with the control (C) rate are shown in the bar graph. The rate constants were calculated from the fit of the efflux data to a first-order rate equation.

because of their primary deficit in cAMP-activated Cl^- efflux. However, the shark rectal gland has been shown to exhibit adenosine inhibition of forskolin-activated Cl^- efflux through an A_1 receptor (31). In addition, adenosine has been shown to activate a 305-pS Cl^- channel in a rabbit cortical collecting duct cell line by action on an A_1 receptor and subsequent activation of protein kinase C (32). Thus, A_1 receptors have been shown to be able to access Cl^- transport in some non-CF elasmobranch and mammalian systems. In the shark, non-cAMP-dependent pathways may also occur.

A separate mechanism for Cl^- efflux, studied in both isolated intestine (33) and T84 cells (34), involves secretion of Cl^- through apical Cl^- channels in response to acetylcholine action at muscarinic receptors. The mechanism involves initial uptake of Cl^- by the $Na^+/K^+/2Cl^-$ cotransporter, activation of basolateral K^+ channels, and consequent development of a hyperpolarizing driving force. The second messenger in this system is probably intracellular Ca^{2+} (34) and additional unidentified factors (35). CFPAC cells could well have such an intact mechanism, although we have no evidence that A_1 receptor antagonists could access this process. Similarly, it is possible that the A_1 receptor antagonists could affect CFPAC cells through volume-sensitive Cl^- channels, such as those reported in T84 cells (36). However, we do not have evidence or reason to expect osmotic gradients in our experiments, nor do we have evidence that swelling occurs in terms of increased chloride space in CFPAC or control cells. In summary, it is not yet evident how the A_1 receptor antagonists effect Cl^- efflux from CFPAC cells.

However, the concept of using xanthine drugs to ameliorate CF symptoms has a long clinical history, inasmuch as theophylline (see Fig. 2) is often given to enhance lung function in asthmatic and CF patients. The mechanism of

theophylline action has been shown to involve both phosphodiesterase inhibition and adenosine antagonism. Therefore, from this perspective, the use of a selective A_1 adenosine receptor-antagonist drug to treat CF would have medicinal precedent in these patients, although the purpose would now be different. The mixed antagonist IBMX (see Fig. 2) has recently been shown to activate Cl^- currents and transport in *Xenopus* oocytes (37) transfected with both *CFTR* and mutated *CFTR* (Phe-508 deletion), although quite high IBMX concentrations (*ca.* 5 mM) were needed to activate the mutant. Similar transfection experiments in VERO cells with lower concentrations of IBMX (10 μM) and a cAMP-elevating cocktail (38) yielded similar results. However, whether or not the A_1 receptor mechanism applies to these latter experiments cannot be discerned from the data presently available. Nonetheless, regardless of the mechanism, the fact that the *CFTR* deletion mutant can be activated is consistent with our recent observations that the recombinant nucleotide-binding fold 1 from CFTR, both wild-type and bearing the Phe-508 deletion mutation, conducts anions across planar lipid bilayers in an ATP-regulated manner (39).

We were also encouraged to find the pharmacological literature fairly replete with studies of CPX action *in vivo*, showing limited intrinsic toxicity. For example, treatment of rats with CPX in the dose range of 0.1 mg/kg of body weight has been reported to be without effect on renal function or morphology. Furthermore, CPX was shown to prevent death caused by glycerol-induced acute renal failure (ARF) over a 2-day or 7-day period (40). CPX also has been shown to reverse adenosine-inhibited gastrin secretion from primary cultures of canine antral cells (41) and to attenuate adenosine-induced bradycardia, but not hypotension, in conscious rats (42). CPX, in the dose range of 30–100 nM, also has been reported to induce a spontaneous burst discharge of Cl^- current in guinea pig hippocampal C3 neurons in brain slices (43). Finally, a close analogue of CPX (KFM 19, Fig. 2) has been tested extensively in lower animals as a cognitive enhancer for treatment of Alzheimer disease (25). Essentially, CPX is a compound with very low apparent toxicity and thus has much to recommend it for further study in terms of efficacy and safety as a drug with possible use for treatment of CF.

We thank Dr. Jack Cohen, Dr. Tilak Raj, Dr. Krishna Ghosh, Dr. A. Lee Burns, Dr. Gemma Kuijpers, Dr. N. Merezhinskaya, Dr. L. Vergara, and Dr. E. Rojas for valuable discussions during the course of this work and Mr. Hung Caohuy for excellent help in some technical aspects of this work. We also thank summer students Ms. Linda Phlairr and Mr. Michael Goldman for their enthusiastic help. This work was supported in part by the Cystic Fibrosis Foundation. During the initial phase of this work, Z.I.C. was on sabbatical leave under the sponsorship of the Cystic Fibrosis Foundation and acknowledges further support by National Institutes of Health Grant HL-40518.

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