8-Cyclopentyl-1,3-dipropylxanthine and Other Xanthines Differentially Bind to the Wild-Type and ΔF508 Mutant First Nucleotide Binding Fold (NBF-1) Domains of the Cystic Fibrosis Transmembrane Conductance Regulator

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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 conductance regulator (CFTR). Certain alkyl xanthines such as CPX (8-cyclopentyl-1,3-dipropylxanthine) stimulate Cl^- efflux from cells bearing the ΔF508 genotype common to most cases of cystic fibrosis. We have hypothesized that the CFTR molecule itself might be the site for CPX action, perhaps in the region of the first nucleotide binding fold (NBF-1) domain. Therefore, to test this hypothesis directly we have used a rapid membrane filtration assay to measure the kinetics of association and dissociation of [^{3}H]CPX to both recombinant NBF-1 and recombinant NBF-1 bearing the ΔF508 mutation. We report that [^{3}H]CPX binds with higher affinity to the ΔF508-NBF-1 of CFTR (K_d = 1.0 nM) than to the wild-type NBF-1 of CFTR (K_d = 17.0 nM). These K_d values were calculated from direct measurements of the association and dissociation rate constants. The rate constants for the dissociation reaction of the wild-type NBF-1 and ΔF508-NBF-1 of CFTR were not different from each other. However, the corresponding rate constants for the association reaction were k_{+1} (NBF-1) = 4.7 ± 0.9 × 10^4 M^{-1} s^{-1} and k_{+1} (ΔF508-NBF-1) = 1.6 ± 0.3 × 10^5 M^{-1} s^{-1}, respectively. These K_d values were corroborated by equilibrium-binding experiments, which gave very similar values. We have also measured the relative displacement of various xanthines from both wild-type NBF-1 and ΔF508-NBF-1, in anticipation that the order of potencies for binding might parallel the action of the different xanthines on CF cells. For wild-type NBF-1, the rank order was DA-CPX > DAX > CPX > caffeine > adenosine >> IBMX > 2-thioCPX. For ΔF508-NBF-1, the rank order was DAX > CPX > caffeine > DA-CPX > adenosine >> IBMX > 2-thioCPX. These relative potencies show close parallels with previously observed relative potencies of these drugs on CF cells, and thus lend strong support to the hypothesis that the mechanism of action on CF cells may involve direct interaction with the CFTR molecule itself.

Cystic fibrosis (CF) is an autosomal recessive disorder affecting chloride transport in pancreas, lung, and other tissues, which in the majority of cases are due to the ΔF508 mutation in the first nucleotide binding fold (NBF-1) of the cystic fibrosis transmembrane regulator (CFTR) (*1*). It has been shown that pancreatic CFPAC cells bearing the CFTR (ΔF508) mutation respond to CPX (8-cyclopentyl-1,3-dipropylxanthine) with an increased Cl^- efflux (*2*−*4*). CPX was also found to enhance Cl^- efflux from IB3 cells derived from the tracheal epithelium of a ΔF508 CF patient. Similar results with CPX have also been reported by others (*5*,*6*). As found for the CFPAC cells, repair with the wild-type CFTR rendered the highly activated Cl^- efflux from the cells relatively insensitive to the xanthine (*3*). Selective enhancement of Cl^- efflux by CPX does not appear to depend on the specific cell type used since mouse fibroblast 3T3 cells expressing either human CFTR or human CFTR (ΔF508) products also exhibited differential effects of CPX on Cl^- efflux (*3*).

CPX is known to act as a very potent antagonist of A_1 adenosine receptors (*7*), and it had been possible that such receptors might be responsible for CPX activation of Cl^- efflux from CF cells. However, the structure−activity relationship of xanthines for the activation of Cl^- efflux from human CFPAC cells did not parallel typical A_1 receptor pharmacology (*4*). Thus, among 26 different xanthines tested, only four were effective agonists of Cl^- efflux from CF cells. The most potent compounds were CPX and DAX (1,3-diallyl-8-cyclohexylxanthine), while 2-thioCPX and IBMX (3-isobutyl-1-methylxanthine) were relatively inactive (*4*). Such an abrupt departure of the structural requirements for xanthines in stimulating Cl^- efflux, compared to the structure−activity relationships of these compounds at A_1 and other subtypes of adenosine receptors (*4*,*8*), suggested a novel binding site for xanthine action, possibly located on the CFTR molecule itself. Supportive evidence for a non-A_1 receptor site of action of CPX on CFPAC cells came from a Northern analysis indicating that A_1 receptor mRNA was missing from these cells (*4*).

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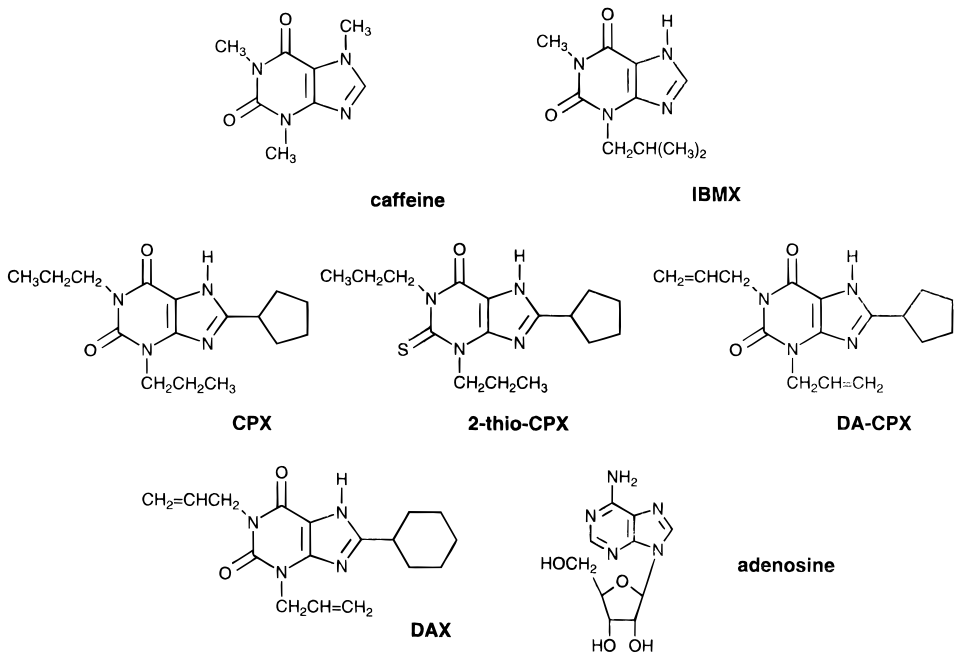

FIGURE 1: Structure of xanthines homologues and adenosine.

To test this hypothesis directly, we first searched the CFTR sequence for any region having homology with the putative CPX interaction site on A_1 and A_2 receptors (*9,10*). The MACAW program (*11*) delineated a segment within the NBF-1 domain near F508 (*12*). This result, plus the importance of F508 in CPX action on CF cells, leads us to test recombinant NBF-1 and ΔF508-NBF-1 for their ability to bind CPX and other xanthines.

In the present work, we have measured the kinetics of association and dissociation of [^{3}H]CPX with both recombinant NBF-1 and recombinant NBF-1 bearing the ΔF508 mutation. In addition, we compared these values with an equilibrium-binding isotherm measured directly using these recombinant molecules. We also measured the ability of CPX and closely related xanthines such as DAX, DA-CPX-(1,3-diallyl-8-cyclopentylxanthine), caffeine, IBMX, and 2-thioCPX to displace [^{3}H]CPX binding from wild-type and mutant NBF-1. The results obtained are consistent with the existence of a unique binding site for CPX and related xanthines in the vicinity of F508. We suggest that this site may play a fundamental role in the mechanism by which CPX activates Cl^- efflux from cells expressing the CFTR (ΔF508) phenotype.

MATERIALS AND METHODS

Materials. Caffeine, IBMX, 2-thio-CPX, and adenosine were purchased from Sigma Chemica Co. (St. Louis, MO). All chemical reagents for synthesis of DAX and DA-CPX were obtained from Aldrich (St. Louis, MO). The structures of the purine derivatives used in this work are shown in Figure 1.

General Procedure for Preparation of 1,3-Diallyl-8-cycloalkylxanthines. To a suspension of 6-amino-1,3-diallyl-5-nitrosouracil (*13*) (47 mg, 0.2 mmol) in 2 mL of ethyl acetate was added a solution of sodium hydrosulfite (286 mg, 1.4 mmol) in 1 mL of water with vigorous stirring. After 1 h of stirring, the starting material completely disappeared to give more polar product on TLC analysis. The reaction mixture was diluted with ethyl acetate/water mixture, and the product was extracted with ethylacetate three times. The organic layer was dried over anhydrous sodium sulfate and evaporated to give a colorless oil. Monoacylation of the oil (1,3-diallyl-5,6-diaminouracil) was performed with 1-[3-(dimethylamino)propyl]-1-ethylcarbodiimide (21 mg, 0.11 mmol) and cycloalkanecarboxylic acid (0.2 mmol) in 1 mL of anhydrous DMF. After stirring for 3 h at room temperature, the reaction mixture was dried with high vacuum, and 5 mL of 2 N NaOH solution was added. The alkaline solution was refluxed for 1 h, cooled, neutralized with 1 N HCl solution, and extracted with ethyl acetate three times. The organic layer was dried over anhydrous sodium sulfate, evaporated, and purified by p-TLC ($CHCl_3$/MeOH = 50/1) to give a white solid (yield 40−50%).

Analysis. Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer, and spectra were taken in $CDCl_3$. The chemical shifts are expressed as parts per million downfield from tetramethylsilane. Chemical ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer. Synthesized DAX and DA-CPX showed one spot on TLC (MK6F silica, 0.25 mm, glass backed, Whatman Inc., Clifton, NJ).

1,3-Diallyl-8-cyclohexylxanthine (DAX). ^{1}H NMR ($CDCl_3$): δ 1.21−1.47 (4H, m), 1.61−1.78 (2H, m), 1.85−1.89 (2H, m), 2.05−2.24 (2H, m), 2.84−2.92 (1H, m), 4.73 (4H, dd, J = 5.8, 18.3), 5.16−5.31 (4H, m), 5.88−6.05 (2H, m). MS (CI): 315 (M + H), 332 (M + NH_4^+).

1,3-Diallyl-8-cyclopentylxanthine (DA-CPX). ^{1}H NMR ($CDCl_3$): δ 1.69−1.73 (3H, m), 1.84−2.00 (3H, m), 2.12−2.16 (2H, m), 3.20−3.26 (1H, m), 4.73 (dd, 4H, J = 5.8, 19.5), 5.17−5.32 (4H, m), 5.88−6.06 (2H, m). MS (CI): 301 (M + H), 318 (M + NH_4^+).

Recombinant Synthesis and Purification of CFTR NBF-1. The prokaryotic pGEX vector expression system was used to synthesize the wild-type CFTR NBF-1 (amino acids 426−588) and ΔF508-NBF-1 of CFTR, as described previously (*14,15*). Purity was assessed by SDS−PAGE analysis. All

samples used in the present study were virtually identical to that previously described (*14*, *15*).

Kinetic Analysis of the Interaction of [^{3}H]CPX with Wild-Type NFB-1 and ΔF508-NBF-1 Recombinant Protein. The interaction of a ligand and a protein binding site to form a complex can be described by a reversible, second-order reaction (*16*). Thus,

$$L + B \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} LB \quad (1)$$

where L and B are the concentrations for the ligand and protein binding sites, respectively; LB is the concentration of the ligand−protein complex formed.

The rate of formation of LB can be expressed by the equation

$$d[LB]/dt = k_{+1}[L][B] - k_{-1}[LB] \quad (2)$$

which on integration yields

$$[LB] = [LB]_e(1 - \exp(k_{+1}[L] + k_{-1}))t \quad (3)$$

where $[LB]_e$ is the concentration of LB at equilibrium.

In order to evaluate the magnitude of the rate constants for the forward (k_{+1}) and reverse reactions (k_{-1}), experimental conditions were chosen so that the concentration of ligand was much greater than the protein concentration (pseudo-first-order approximation). Thus, in this case [L] >> [B] and

$$B \underset{k_{-1}'}{\overset{k_{+1}'}{\rightleftharpoons}} LB \quad (4)$$

so that $k_{+1}' = k_{+1}$ [L].

Solving eq 3 for the time at which [LB] reaches one-half of its equilibrium value ($t_{1/2}$) and applying the pseudo-first-order approximation, the following equation is obtained:

$$\ln 2/t_{1/2} = k_1[L] + k_{-1} \quad (5)$$

This equation indicates that, by plotting the inverse of the half-times ($\ln 2/t_{1/2}$) corresponding to ligand binding at different L concentrations, we get a straight line with an intercept equal to k_{-1} and a slope equal to k_1. Thus, the ratio of intercept to slope in such plots yields the corresponding equilibrium dissociation constant $K_d = k_{-1}/k_1$.

The kinetic characteristics of the interaction between CPX and the recombinant wild-type and mutant ΔF508-NBF-1 were investigated by measuring the rate at which [^{3}H]CPX binds to the protein at different [^{3}H]CPX concentrations (eq 5). For this purpose, increasing concentrations of [^{3}H]CPX (ICN, SA = 109 Ci/mol) were incubated for different times with wild-type NBF-1 and ΔF508-NBF-1 domains of CFTR (1.0 nM concentration) in 0.1 mL of a 300 mM sucrose solution buffered with Tris at pH = 6.0. Bound and free ligand were separated by rapid filtration through a nitrocellulose membrane (S & S, NC) on a dot blot apparatus. Nonspecific [^{3}H]CPX binding to nitrocellulose filters was reduced by blocking the filters with 0.3% polyethyleneimine (PEI) (*17*). For the corresponding equilibrium determinations of K_d, a constant amount of the recombinant wt-NBF-1 (2.0 nM) was incubated for 1 h with increasing concentrations of [^{3}H]CPX, and then the mixture was filtered through the filtration apparatus. Nonspecific binding was measured by adding an excess of nonradioactive CPX (10 μM). The dot blots were washed three times with 0.2 mL of an ice-cold buffered TRIS-NaCl solutions (50 mM TRIS pH 7.0, 150 mM NaCl). Radioactive spots were cut out, and total radioctivity was determined by liquid scintillation counting. Specific [^{3}H]CPX binding to recombinant protein was determined by subtracting the nonspecific binding (pre-incubation with 10 μM unlabeled CPX) from total binding.

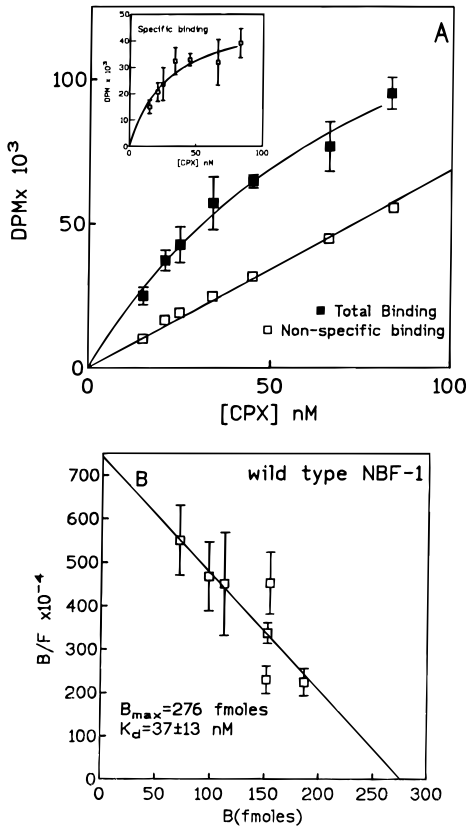

FIGURE 2: (A) Representative saturation curve for binding of [^{3}H]-CPX at recombinant wt-NBF-1 of CFTR, showing nonspecific and total binding. (Inset) Specific binding (in DPM × 10^3). (B) Corresponding Scatchard analysis. A K_d value ± SEM of 37.0 ± 13 nM was obtained (B_{max} = 0.86 ± 0.3 mol/mol of protein).

For displacement studies, different concentrations of nonradioactive CPX, DA-CPX, DAX, 2-thioCPX caffeine, IBMX, and adenosine were incubated with 40 nM [^{3}H]CPX and 2.0 nM of either recombinant NBF-1 or ΔF508-NBF-1 in 0.1 mL of a 300 mM sucrose solution buffered with 50 mM Tris-HCl at pH 6.0. The binding was carried out for 1 h before filtering as described above. All stock solutions of nonlabeled compounds were dissolved in DMSO and diluted in buffer to the final concentration, where the amount of DMSO never exceeded 1%. All assays were done in triplicate.

Data Analysis. Kinetic data were fit to exponential curves by using a nonlinear regression formula on the Graphpad-Inplot program. IC_{50} values were also computed generated from competition on binding data using a nonlinear regression formula in the Graphpad program. IC_{50} values were converted to K_i values by using the Cheng−Prusoff equation (*18*).

RESULTS

[^{3}H]CPX Binding to wt-NBF-1 and ΔF508-NBF-1. A representative saturation isotherm for specific binding of

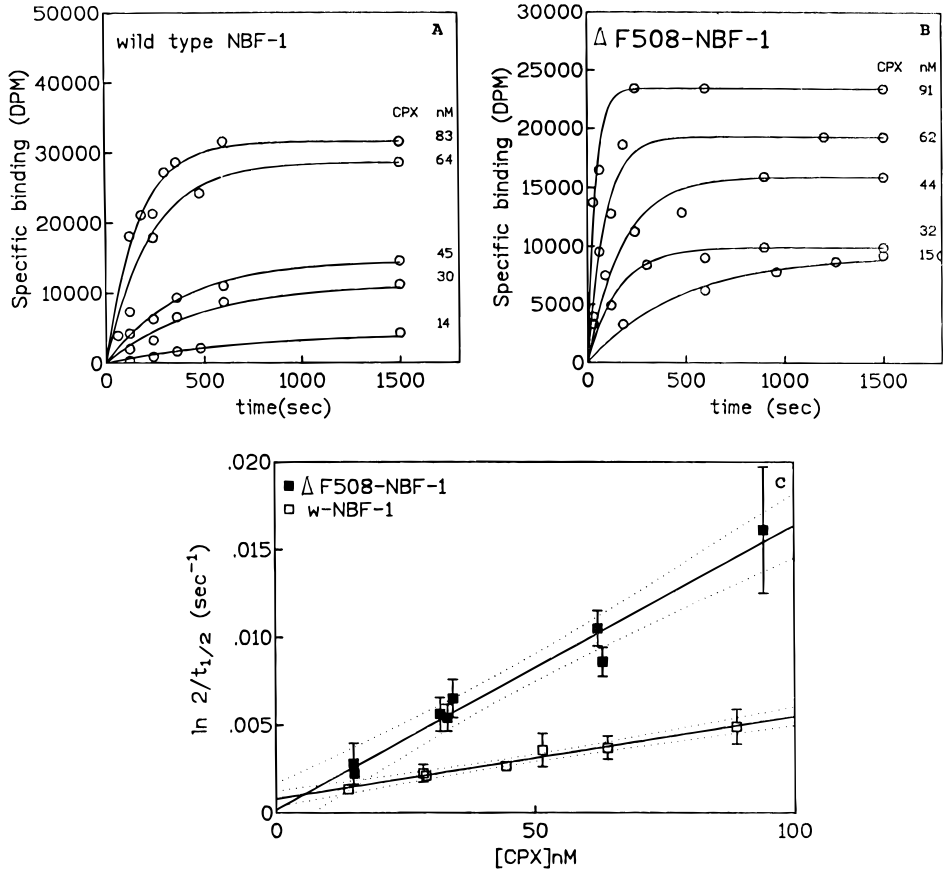

FIGURE 3: Binding reaction for [^{3}H]CPX for varying concentrations of [^{3}H]CPX to recombinant wt-NBF-1 (A) and ΔF508-NBF-1 (B) of CFTR. Varying concentrations of [^{3}H]CPX were added to 0.1 nM recombinant protein in Hepes-sucrose buffer at pH 6.0. At the times indicated, aliquots were rapidly passed over nitrocellulose filters (pore size = 0.45 μm), and ^{3}H was determined on triplicate samples as described in Materials and Methods. (C) Analysis of data in parts A and B. The corresponding plot of ln $2/t_{1/2}$ against varying [^{3}H]CPX concentrations (in nanomolarity). □, wt-NBF-1; ■, ΔF508-NBF-1.

[^{3}H]-CPX to recombinant wt-NBF-1, with nonspecific binding defined in the presence of 10 μM CPX is shown in Figure 2A. The amount of specific binding was approximately 60% of total binding (inset of Figure 2A). A Scatchard plot of the data (Figure 2B) is linear with a K_d = 37.0 ± 13 nM (mean of three separate experiments) and a binding capacity B_{max} = 0.86 ± 0.3 fmol/fmol of protein.

Association kinetics for [^{3}H]CPX to recombinant wt-NBF-1 (Figure 3A) or to ΔF508-NBF-1 (Figure 3B) reached equilibrium within 20 min at all radioligand concentrations tested. The experimental data points were fitted to an exponential curve using GraphPad Inplot iterative curve fitting. The half-times ($t_{1/2}$) obtained for equilibrium CPX binding on both wild-type and mutant proteins were analyzed according to eq 5 (see Materials and Methods). Thus, by plotting ln $2/t_{1/2}$ values against varying [^{3}H]CPX concentrations, straight lines were obtained in both cases (Figure 3C). This indicates the validity of assuming a simple reversible model for CPX binding in this system. The least-squares regression analysis estimate of the *y*-intercepts of these plots was 7.8 ± 4.6 × 10^{-4} s^{-1} for wt-NBF-1 and 1.6 ± 15.0 × 10^{-4} s^{-1} for ΔF508-NBF-1 (95% confidence intervals). Thus, within the limits of error in these experiments, the rate constants for the dissociation reaction k_{-1} of wt-NBF-1 and ΔF508-NBF-1 are not different from each other (see dotted lines in Figure 3C). On the other hand, k_{+1}(wt-NBF-1) = (4.7 ± 0.9) × 10^4 M^{-1} s^{-1} and k_{+1} (ΔF508-NBF-1) = (1.6 ± 0.3) × 10^5 M^{-1} s^{-1} (±95% confidence intervals), respectively. The measured k_{-1} and k_{+1} rate constants give an estimate of the dissociation constant K_d of 17 nM for wt-NBF-1 and a K_d of 1 nM for ΔF508-NBF-1. These values are in good agreement with the K_d values determined from equilibrium experiments (see Figure 2B).

Inhibition of [^{3}H]CPX Binding to Wild-Type NBF-1 and ΔF508-NBF-1 of CFTR by Xanthine Derivatives and Adenosine. We have investigated the ability of various alkyl xanthine derivatives, which have also been tested as enhancers of Cl^- efflux across CFPAC cells, to competitively displace [^{3}H]CPX binding to recombinant wt-NBF-1 and ΔF508-NBF-1. As shown in Figure 4, all the displacement curves for six xanthines used and adenosine also (Figure 4) were monophasic with slope factors approximately equal to unity and correlation coefficients over 0.8. The IC_{50} and corresponding K_i values obtained for CPX and structurally related derivatives are shown in Figure 5 and Table 1.

The unlabeled compound CPX itself (1,3-dipropyl-8-cyclopentylxanthine) was a very potent inhibitor of [^{3}H]CPX binding with a K_i of 23 nM for wt-NBF-1 and a K_i of 0.8 nM for ΔF508-NBF-1. These values are in good agreement with the K_d values derived from equilibrium and kinetic experiments with [^{3}H]CPX (see Figure 2B and Figure 3C). Other xanthines such as DA-CPX and DAX (structures shown in Figure 1) were even more potent than CPX in displacing radioligand bound to wt-NBF-1 and ΔF508-NBF-1, respectively, with K_i values in the nanomolar range (Table 1). Thus, the rank order of affinity for binding was DA-CPX > DAX > CPX > caffeine > adenosine >> IBMX > 2-thio-CPX for wt-NBF-1 and DAX > CPX > caffeine

Table 1: Affinities of Xanthine Derivatives and Adenosine on Recombinant Wild-Type NBF-1 and ΔF508-NBF-1 of CFTR

compound	wt-NBF-1, IC_{50} (nM)	ΔF508-NBF-1, IC_{50} (nM)	wt-NBF-1, K_i (nM)	ΔF508-NBF-1, K_i (nM)	ratio, K_i(wt)/K_i (ΔF508)	threshold stim. of Cl^- eff[a] (nM)	affinity at A_1 receptors K_i (nM)[a]
DAX	66 ± 19	7 ± 1	20 ± 5	0.18 ± 0.02	110	30	65.6
CPX	79 ± 7	33 ± 10	23 ± 2	0.8 ± 0.2	29	1	0.46
DA-CPX	53 ± 12	61 ± 19	16 ± 4	1.5 ± 0.5	11		
caffeine	174 ± 40	43 ± 13	52 ± 12	1.1 ± 0.3	49	1	29 000
adenosine	275 ± 96	201 ± 70	82 ± 29	5 ± 2	17		
IBMX	2600 ± 730	3100 ± 1100	776 ± 220	77 ± 27	10	$>10^4$ [b]	7000
thioCPX	8200 ± 2200	19 000 ± 8000	2400 ± 650	463 ± 200	5	$>10^4$ [b]	0.66

[a] Values taken from ref 4. [b] Concentration of maximal stimulation of Cl^- efflux. IC_{50} value are the mean ± SD of at least two experiments done in triplicate. IC_{50} values were converted to K_i values by using the Cheng−Prusoff equation (*18*) with K_d values of 17 nM and 1 nM for CPX binding to wt-NBF-1 and ΔF508-NBF-1, respectively.

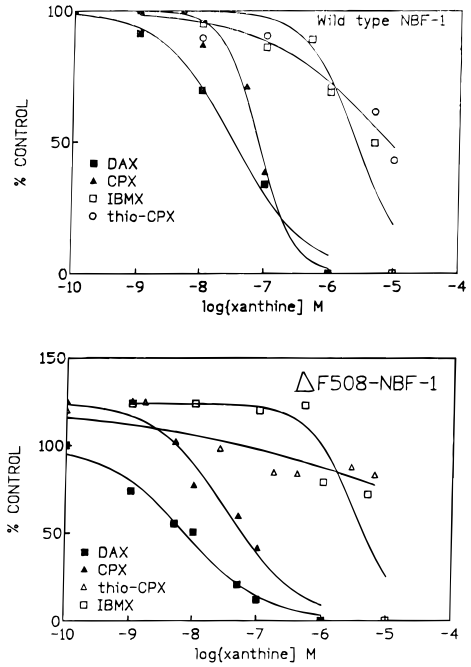

FIGURE 4: Representative competition curves for inhibition of binding of [^{3}H]CPX by different xanthines from wt-NBF-1 (A) and ΔF508-NBF-1 (B) of CFTR, expressed as percent of control specific binding. Each point represents the average ± SEM of three determinations done in duplicate.

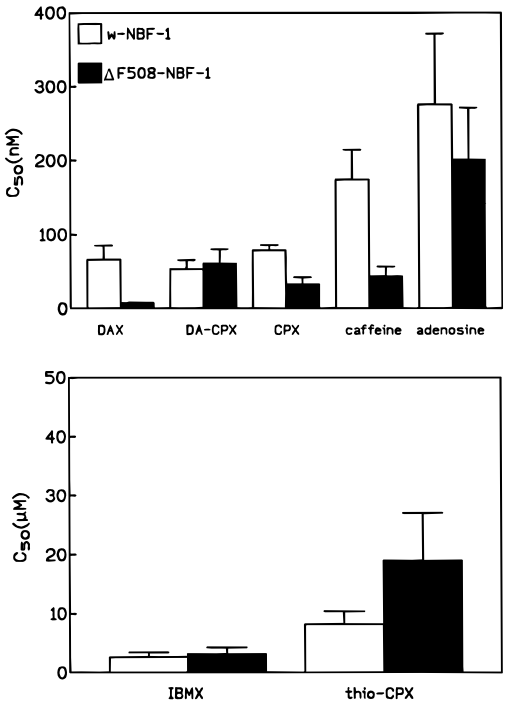

FIGURE 5: Affinities of xanthines and adenosine on wt-NBF-1 and ΔF508-NBF-1 of CFTR, expressed as IC_{50} values.

> DA-CPX > adenosine >> IBMX > 2-thioCPX for ΔF508-NBF-1 (Table 1).

The ratios of K_i values for wt-NBF-1/ΔF508-NBF-1 for different xanthines are shown in Table 1. The xanthines differentially affected the K_i ratios of wild-type over mutant NBF-1. For example, the ratio of K_i values for CPX was 29, whereas for DAX, an 8-cyclohexyl analogue, this ratio increased to 110. Nonetheless, the value of the K_i ratios does not appear to depend only on the presence of an 8-alkyl group. For example, for caffeine (1,3-triimethylxanthine) the ratio was 49, a value that was even greater than that of CPX (Table 1). Yet, as the results in Table 1 indicate, the presence of an oxo group at the 2-position and dipropyl groups at the 1- and 3-positions, appear to be essential because 2-thioCPX (8-cyclopentyl-1,3-dipropyl-2-thioxanthine) and IBMX have ratios of only 5 and 10, respectively (Table 1), and have relatively poor affinity.

The results shown in Table 1 also indicate that adenosine can displace bound [^{3}H]CPX from wild-type and mutant ΔF508-NBF-1 with K_i values of 82 and 5 nM, respectively. As can also be noted from Table 1, the affinity of adenosine at wt-NBF-1 is weaker than most of the xanthines investigated, with the exception of IBMX and thioCPX. The ratio of K_i values for adenosine (for wt-NBF-1 over ΔF508-NBF-1) was 17.

DISCUSSION

The present results demonstrate that CPX binds with high affinity to both the wt-NBF-1 and mutant ΔF508-NBF-1 of CFTR. Furthermore, both kinetic and equilibrium binding analysis of CPX are in fundamental agreement as to the binding constants. Interestingly, these values are similar to those found for CPX activation of Cl^- efflux of cells bearing the CFTR(ΔF508) mutation (*2*−*4*). In addition, our results indicate that the xanthine derivatives display distinct patterns in displacing CPX bound to both wild-type NBF-1 and ΔF508-NBF-1 of CFTR (Table 1). Such patterns resemble the rank order of potencies found for the activation of Cl^- efflux from cells bearing the CFTR (ΔF508) mutation (Table 1). Thus, the lack of activity observed for the 2-thioCPX derivative in both wild-type and mutant NBF-1 (Table 1) parallels the observation that maximal stimulation of Cl^- efflux by this compound on CFPAC cells is obtained at concentrations substantially greater than 1 μM (*4*). Consistently, caffeine exhibits a relatively high binding affinity both

for wt-NBF-1 as well as for ΔF508-NBF-1 (Table 1). In fact, the K_i value measured for the binding of this compound to ΔF508-NBF-1 is identical to the threshold concentration required to stimulate Cl^- efflux (Table 1). By contrast, these affinities are entirely different from those of the same compounds binding at A_1 adenosine receptors (*19*). These data thus lend further support to the concept that the mechanism of action of CPX on cells bearing the CFTR (ΔF508) mutation may involve direct action on a unique binding site for xanthines located in the NBF-1 domain of CFTR. This concept further suggests that involvement of adenosine A_1 or other subtypes of adenosine receptors, is not necessary for the specific activation process.

Molecular Parameters Determining the Interaction of Xanthines with the Wild-Type and Mutant Forms of the NBF-1. The virtual inactivity of 2-thioCPX in displacing CPX bound to wt-NBF-1 and ΔF508-NBF-1 reveals the possible involvement of the 2-position of the xanthine in H-bonding to an acceptor group at the binding site. No such involvement has been proposed for the interaction of CPX and other xanthines derivatives with A_1 receptors, since in such a system no loss of activity of 2-thio derivatives has been found (*8*). In this respect, the fact that caffeine appears to interact with an affinity comparable to CPX at the binding site of ΔF508-NBF-1, may be taken as an indication that the absence of an 8-alkyl group substitutent in this compound is presumably overcome by a decreased interference of the 1,3-dimethyl groups with H-bonding to the 2-oxo group of caffeine. Such an interference by the more bulky alkyl groups present at the 1- and 3-positions of the xanthine may also explain why IBMX, a 1-methyl-3-isobutyl xanthine, is weakly active in displacing CPX bound to both wild-type and mutant NBF-1 (Table 1).

The present observation of a greater affinity of CPX and other xanthines to ΔF508-NBF-1, as compared to the wild-type NBF-1 (Table 1), could be taken as an indication that the absence of F508 in the mutant recombinant protein, allows for a local change in the conformation of the xanthine binding site. Thus, the ratio of wt-NBF-1 over ΔF508-NBF-1 was about 10-fold greater for DAX (a 8-cyclohexyl analogue) than for DA-CPX (a 8-cyclopentyl analogue) (Table 1).

The magnitude of such an enhancement is greater than the 3.6-fold increment that can be calculated for the octanol/water partition coefficient of a xanthine having a cyclohexyl group rather than a cyclopentyl group (*20*). This result suggests that a more lipophilic pocket is formed at the site in ΔF508-NBF-1, which is interacting with the 8-position of the xanthine. Such a localized conformational change cannot be directly demonstrated until a three-dimensional structure of the wild-type NBF-1 and mutant forms are available.

However, recent studies of the fluorescence emission spectra of the wild-type and ΔF508-NBF-1 reveal a significant blue-shift several nanometers from the peak fluorescence of the mutant (*21*). This observation is consistent with the single tryptophan at position 496 (W496) being surrounded by a more hydrophobic environment. Indeed, previously, spectroscopic studies of peptide models of NBF-1 consisting of 67 or 66 amino acids spanning the region from R450 to P516 of CFTR have indicated that the absence of F508 increased the peptide sensitivity to denaturating conditions.

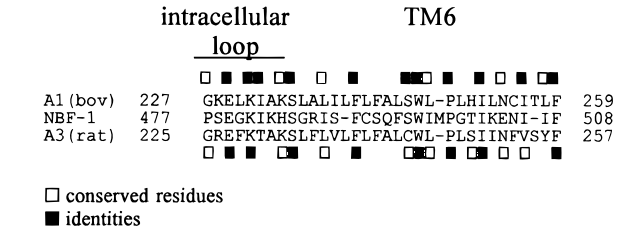

FIGURE 6: Sequence homologies between NBF-1 of CFTR and adenosine A_1 and A_3 receptors. Amino acid residues 477−508 of the NBF-1 of CFTR were aligned using the Macaw program (*11*) with the sequences of bovine A_1 adenosine receptor and rat A_3 adenosine receptor. Identical or homologous residues between NBF-1 of CFTR and A_1 or A_3 adenosine receptors are indicated. The definition of homology is that of the Swiss-Prot data bank, in which amino acids in the following groups are homologous: [S, T, A, G, P]; [N, D, E, Q]; [R, K, H]; [M, L, I, V], and [F, Y, W].

This finding can be also be taken as evidence of an increased exposure of hydrophobic groups (*22*, *23*).

Xanthine Binding Site Location in NBF-1. Concerning the problem of locating the xanthine binding site within the NBF-1 of CFTR, it is important to note that adenosine was also found to displace [^{3}H]CPX bound to NBF-1 and ΔF508-NBF-1 (Table 1). It was therefore not surprising that a homology could be found within the NBF-1 sequence for authentic A_1 adenosine receptors. Thus, by using the multiple protein sequence alignment program MACAW (*11*), we found a significant homology between adenosine receptors and a single region of the NBF-1 located in the vicinity of F508 (Figure 6). The sequences for the A_1 and A_3 receptors span the distance from the distal region of the third intracellular loop to the sixth transmembrane domain (TM6). The coaligned NBF-1 sequence of CFTR extends from P477 to F508. In order to optimize the number of identities and conserved residues in the alignment of NBF-1 with A_1 receptors shown in Figure 6, one gap in the A_1 sequence and two gaps in the CFTR sequence need to be introduced. It can be observed that the first nine positions of the alignment shown are occupied mostly by hydrophilic residues including E229, K231, K234, and S235 of the A_1 receptor. These align with E479, K481, H484, and S485, respectively of the NBF-1 of CFTR (identities are underlined). None of these residues in A_1 receptors have been shown to be involved in ligand binding but may act as membrane anchors by interacting with the phospholipid bilayer (*24*). However, TM6 of the A_1 receptor, which starts at K234 and the homologous sequence of the A_{2a} receptor, is a region prominently involved in xanthine and adenosine binding, particularly by amino acid residues such as H251 and N254. Mutations of these key residues have indicated reduced ligand binding (*9*,*10*). There is a total number of 11 identical residues and six conserved residues spanning a 32 amino acid portion of NBF-1 of CFTR (i.e., 50% of total residues are conserved). Such homology may provide the rationale for our preliminary observation that a synthetic peptide sequence of 32 residues, referred to as Iα or CFTR (477−508), also binds CPX specifically and with high affinity (*25*).

According to the alignment shown in Figure 6, another property that can be anticipated for the Iα peptide and its parent NBF-1 of CFTR is the intrinsic capacity to interact with phospholipid membranes. This prediction has also been born out by experiments which demonstrated that Iα and both wild-type and ΔF508-NBF-1 were able to induce the aggregation of PS liposomes and chromaffin granules in a

Ca^{2+}-dependent manner (*25*,*26*) and the formation of ion channels across planar lipid bilayers (*15*).

We can conclude from the present study that a putative protein target for the xanthine CF drugs has been found. Future biophysical studies of the interaction of CPX, DAX, and other potent stimulators of Cl^- efflux with NBF-1 may result in improved design of agents for chemical rescue of malfunctional CFTR.

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