Stimulation by Alkylxanthines of Chloride Efflux in CFPAC-1 Cells Does Not Involve A_1 Adenosine Receptors†

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ABSTRACT: A series of 8-substituted derivatives of 1,3,7-alkylxanthines was synthesized as potential activators of chloride efflux from a human epithelial cell line (CFPAC) expressing the cystic fibrosis transmembrane regulator (CFTR) ΔF508 mutation. Their interactions with rat brain A_1 and A_{2a} receptors were also studied in radioligand binding experiments. Substitution was varied at the xanthine 1-, 3-, 7- and 8-positions. 1,3-Dipropyl-8-cyclopentylxanthine (CPX) stimulated Cl^- efflux in the 10^{-8} M range, with a maximal effect reaching 200% of control and diminishing at higher concentrations. The potent adenosine antagonist 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, nonselective at human A_1 and A_{2a} receptors, was inactive in Cl^- efflux. 1,3-Diallyl-8-cyclohexylxanthine (DAX) was highly efficacious in stimulating chloride efflux with levels reaching >300% of control, although micromolar concentrations were required. 1,3,7-Trimethyl-8-(3-chlorostyryl)xanthine, an A_{2a}-selective adenosine antagonist, was only weakly active. Caffeine, which acts as an nonselective adenosine antagonist in the range of 10^{-5} M, was active in Cl^- efflux in the low nanomolar range but with low efficacy. Thus, among the xanthine derivatives of diverse structure, there was no correlation between potency in Cl^- efflux and adenosine antagonism. Poly(A)+ RNA isolated from CFPAC-1 cells showed no hybridization to a human A_1 receptor cDNA probe, excluding this receptor as a mediator of CPX-elicited Cl^- efflux. Thus, this action of xanthines in stimulating Cl^- efflux in CFPAC cells, which express a defective CFTR, represents a novel site of action apparently unrelated to adenosine receptors.

Cystic fibrosis is the most common genetic disease among the Caucasian population (Boat et al., 1989) and is characterized by a defect in the 3′,5′-cyclic AMP-activated chloride efflux from affected epithelial cells (Welsh, 1990; Quinton, 1990) and by abnormal mucin secretion in the lungs, intestines, and other affected organs (Boat et al, 1989). The molecular basis of this disease has been traced to specific mutations in the cystic fibrosis transmembrane regulator (CFTR)[1] gene (Riordan et al. 1989; Rommens et al., 1989; Karem et al., 1989), which encodes a specific cyclic AMP-activated anion channel activity in the apical membranes of cells from affected organs (Bear et al., 1992). The mutation accounting for more than 70% of cystic fibrosis cases is the deletion of Phe^{508} (ΔF508) in the first nucleotide binding fold (NBF-1) domain. The consequence of the ΔF508 mutation is that, while the channel is intact in overall structure (Drumm et al., 1991; Dalemans et al., 1991; Anderson et al., 1991; Arispe et al., 1992), the mutant gene product may not be trafficked correctly in the apical membrane to be active (Chang et al., 1990; Team et al., 1993; Kartner et al., 1992). ATP-gated chloride channels can be detected when the recombinant NBF-1 domains containing either the wild type or ΔF508 mutation are incorporated into planar lipid bilayers (Arispe et al., 1992).

We have shown that CPX (8-cyclopentyl-1,3-dipropylxanthine), a xanthine derivative that is a potent A_1 adenosine antagonist, promotes chloride efflux from a human epithelial cell line (CFPAC-1) expressing the CFTR ΔF508 mutation (Eidelman et al, 1992). The mechanistic explanation for this action of CPX has not been apparent from consideration of the second messengers to which the adenosine receptors are known to be coupled. Inhibition of cyclic AMP results from activation of A_1 receptors, and phosphoinositide breakdown and other second messengers are also involved in certain tissues [reviewed in Van Galen et al. (1992)]. The insensitivity to cyclic AMP by the CFTR-based chloride efflux system is one of the hallmarks of cystic fibrosis. The possible relationship to G_i-protein activation by CPX acting at A_1 receptors has also been explored in cell lines derived from human lung, and the potency of CPX on activation of outward chloride current has been confirmed (Schwiebert et al., 1992; Haws et al., 1994) In our own laboratory (H.B.P.), CPX stimulation of chloride efflux has been detected in two CF cell types bearing the ΔF508 mutation,

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[1] Abbreviations: CFTR, cystic fibrosis transmembrane regulator; CGS 21680, 2-[[[4-(2-carboxyethyl)phenyl]ethyl]amino]-5′-(*N*-ethylcarboxamido)adenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CSC, 8-(chlorostyryl)caffeine; DAX, 1,3-diallyl-8-cyclohexylxanthine; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDE, phosphodiesterase; PIA, (*R*)-N^6-(phenylisopropyl)adenosine; SAR, structure−activity relationships; Tris, tris(hydroxymethyl)aminomethane; XAC, 8-[[[[[(2-aminoethyl)amino]carbonyl]methyl]-oxyphenyl]-1,3-dipropylxanthine.

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pancreatic CFPAC-1 cells and lung IB3 cells. Curiously, the stimulation of Cl^- efflux by CPX occurs only in the cells expressing the defective CFTR protein and not after correction of the cystic fibrosis defect by retrovirus-mediated gene transfer (Drumm et al, 1990; Guay-Broder et al., 1995).

In this study we have characterized the highly restricted structural requirements of 1,3-dialkyl- and 1,3,7-trialkylxanthines as activators of chloride efflux in CFPAC cells. We were especially drawn to this analysis because this activity is thought to be predictive of potential therapeutic agents for treatment of cystic fibrosis (Eidelman et al, 1992). Our principal finding was that the structural requirements for xanthines in stimulating chloride efflux do not correlate with well-described structure−activity relationships at the known subtypes of adenosine receptors (Jacobson et al., 1992, 1993a; Shamim et al., 1989; Shimada et al., 1992). This is suggestive of a novel mechanism and involvement of a unique binding site.

EXPERIMENTAL PROCEDURES

Chemistry. Compounds **8**−**12**, **14**, **18**, **19**, **20**, and **25** were synthesized as described (Jacobson et al., 1988, 1989; Shimada et al., 1992; Shamim et al., 1989). Compounds **1**−**5**, **26**, and 2-chloroadenosine were purchased from Research Biochemicals International (Natick, MA). Compounds **7**, **14**, **18**, and **19** (Müller et al., 1993; Shamim et al., 1989) were the gift of Dr. John W. Daly, Dr. Christa Müller, and Dr. Mah Shamim (NIH). Compound **27** (Thompson et al., 1991) was the gift of Dr. Ray Olsson (University of South Florida, Tampa, FL).

Several new 8-substituted xanthines were synthesized by standard methods (Shamim et al., 1989). An alkyl- or cycloalkylcarboxylic acid was condensed with 5,6-diamino-1,3-dimethyluracil to obtain the corresponding 6-amino-5-carboxamido-1,3-dialkyluracil derivative. This procedure was used with cyclohexylcarboxylic acid, cyclohexylacetic acid, and cycloheptylcarboxylic acid to synthesize compounds **17**, **21** (mp 236−238 °C), and **23** (mp 223−225 °C), respectively.

Alkylation of the N-7 position of xanthine derivatives was carried out as described (Jacobson et al., 1993a) in dimethylformamide/potassium carbonate. This procedure was used with cyclopropylmethyl iodide (**15**) and ethyl iodide (**16**) and with methyl iodide to give compounds **22** (mp 158−159 °C) and **24** (mp 199−201 °C).

Biological Methods: Receptor Binding. The analogs were tested in a radioligand binding assay for affinity to adenosine receptors in membranes. The compounds were assayed for affinity to rat brain A_1 receptors using [^{3}H]-N^6-(phenylisopropyl)adenosine (Schwabe & Trost, 1980) and at rat striatal A_{2a} receptors using [^{3}H]CGS 21680 (Jarvis et al., 1989). Rat cerebral cortical membranes and striatal membranes were prepared (Jacobson et al., 1993a) and treated with adenosine deaminase (2 units/mL) for 30 min at 37 °C prior to storage at −70 °C. Solid samples of the adenosine derivatives were dissolved in DMSO and stored in the dark at −20 °C. The stock solutions were diluted with DMSO to a concentration of ≤0.1 mM prior to addition to the aqueous medium. The final concentration of DMSO in the assay medium was generally 2%. Inhibition of binding of 1 nM [^{3}H]-N^6-(phenylisopropyl)adenosine (Dupont NEN, Boston, MA) to A_1 receptors in rat cerebral cortex membranes was measured as described (Schwabe & Trost, 1980; Jacobson et al., 1993a). Membranes (~100 μg of protein/tube) were incubated for 1.5 h at 37 °C in a total volume of 0.5 mL of 50 mM Tris hydrochloride, at pH 7.4. Test drugs were dissolved in DMSO and added in 10 μL aliquots, resulting in a final DMSO concentration of 2%. Bound and free radioligand were separated by addition of 3 mL of a buffer containing 50 mM Tris hydrochloride, at pH 7.4 at 5 °C, followed by vacuum filtration using a Brandel cell harvester (Brandel, Gaithersburg, MD) and a Whatman GF/B glass fiber filter with additional washes totaling 9 mL of buffer. Nonspecific binding was determined with 10 μM 2-chloroadenosine.

Inhibition of binding of 5 nM [^{3}H]CGS 21680 (2-[[[4-(2-carboxyethyl)phenyl]ethyl]amino]-5′-(*N*-ethylcarboxamido)-adenosine, Dupont NEN, Boston, MA) was carried out as reported (Jarvis et al., 1989). Membranes (~80 μg of protein/tube) were incubated for 1 h at 25 °C in a total volume of 0.5 mL of 50 mM Tris hydrochloride containing 10 mM $MgCl_2$ at pH 7.4. Test drugs were dissolved in DMSO and added in 10-μL aliquots, resulting in a final DMSO concentration of 2%. Nonspecific binding was defined using 20 μM 2-chloroadenosine. Filtration was carried out using a Brandel cell harvester, as above, using Tris-HCl/$MgCl_2$ as the washing buffer.

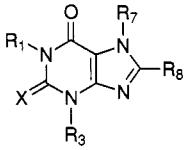
At least six different concentrations spanning 3 orders of magnitude, adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, computer-generated using a nonlinear regression formula on the GraphPAD program (Institute for Scientific Information), were converted to apparent K_i values using K_D values (Jacobson et al., 1993a; Ukena et al. 1986) of 1.0 and 14 nM for [^{3}H]PIA and [^{3}H]-CGS 21680 binding, respectively, and the method of Cheng and Prusoff (1973).

Measurement of $^{36}Cl^-$ Fluxes. The effect on chloride efflux in CFPAC cells was measured directly (Eidelman et al., 1992) in culture wells containing cells preloaded with radioactive chloride.

CFPAC-1 cells were obtained in 1992 from Dr. R. Frizzell, University of Alabama at Birmingham. The cells were grown in Iscove's medium, supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/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.

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 Na[^{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 equilibrium. To initiate efflux experiments, appropriate concentrations of CPX or other xanthines were added to the cells and incubated for 15 min at 19 °C. This temperature was chosen to optimize the rate of efflux for the purpose of multiple sampling. The wells were then washed four times with ice-cold wash medium, containing 150 mM sodium gluconate, 1.5 mM potassium gluconate, and 10 mM Na-HEPES, pH 7.4. The drug was also present during the washing cycles.

Table 1: Activity of Xanthine Derivatives as Adenosine Antagonists[a] and in the Rate of Chloride Flux in CFPAC Cells[b]



compd[c]	R_1, R_3 =	R_7 =	R_8 =	K_i, μM (A_1)	K_i, μM (A_2)	max stimulation (nM)	% cntrl	threshold stimulation (nM)	% cntrl
			8-Unsubstituted						
1	Me	H	H	8.5	25	100	150 ± 30	1	130 ± 20
2	Me	Me	H	29	48	10	170 ± 20	1	120 ± 30
3	Me, *i*-Bu[d]	H	H	7	16	$\gg 10^4$			
			8-Cyclopentyl						
4	Me	H	cyclopentyl	0.011	1.4	$\gg 10^4$			
5[e]	Pr	H	cyclopentyl	0.00046	0.34	30	200 ± 10	1	130 ± 10
6	Pr	Me	cyclopentyl	2.3	11.4 ± 1.4	1000	180 ± 40	30	125 ± 30
7	Pr, H[f]	H	cyclopentyl	0.014	0.58	30	160 ± 40	1	130 ± 20
8[g]	Pr	H	cyclopentyl	0.00066	0.31	$\gg 10^4$			
9	Pr	H	3-fluorocyclopentyl	0.042		$\gg 300$			
10	Pr	H	3-iodocyclopentyl	0.058		$\gg 300$			
11	Pr	H	cyclopenten-3-yl	0.045	0.640 ± 0.061	$\gg 300$			
12[h]	Pr	H	noradamantyl	0.0013	0.38	10	170 ± 20	1	120 ± 5
			8-Cyclohexyl						
13	Me	H	cyclohexyl	0.030 ± 0.015	1.04 ± 0.20	3	160 ± 20	0.1	130 ± 20
14	Me	Me	cyclohexyl	28	17.1	3	150 ± 30	3	150 ± 30
15	Me	Et	cyclohexyl	6.66 ± 0.99	3.23 ± 0.36	$\gg 10^4$			
16	Me	(cyclopropyl)-methyl	cyclohexyl	8.23 ± 1.62	4.53 ± 1.37	$\gg 10^4$			
17[k]	allyl	H	cyclohexyl	0.0656 ± 0.0189	4.94 ± 0.68	10^3	260 ± 70	30	170 ± 40
18	Pr	H	cyclohexyl	0.0015	0.423 ± 0.055	$\gg 10^4$			
19	Pr	Me	cyclohexyl	2.7	9.24 ± 0.37	5	170±50	5	170 ± 50
20	Pr	H	cyclohexen-3-yl	0.010	0.905 ± 0.128	10^4	150±30	10^4	150 ± 30
21	Me	H	cyclohexylmethyl	0.610 ± 0.076	5.47 ± 0.86	$\gg 10^4$			
22	Me	Me	cyclohexylmethyl	3.71 ± 0.36	5.46 ± 0.47	$\gg 10^4$			
			8-Cycloheptyl						
23	Me	H	cycloheptyl	0.0659 ± 0.0145	1.05 ± 0.19	$\gg 10^4$			
24	Me	Me	cycloheptyl	19.5 ± 2.5	3.27 ± 0.98	$\gg 10^4$			
			8-Stryryl or 8-Aryl						
25[i]	Me	Me	3-chlorostyryl	28	0.054	30	160 ± 30	30	160 ± 30
26[j]	Pr	H	*p*-(C_6H_4)-OCH_2CONH-$(CH_2)_2NH_2$	0.0012	0.08	$\gg 10^4$			
			Non-Xanthine Antagonist						
27		9-ethyl-6-cyclopentyladenine		0.44	17	$\gg 10^4$			

[a] Values are given ± SEM, except for literature values taken from Müller et al. (1993), Jacobson et al. (1985, 1988, 1989, 1992, 1993a,b), Shamim et al. (1989), Shimada et al. (1992), Thompson et al. (1991), and van Galen et al. (1992). A_1 and A_{2a} affinities were measured in rat brain using [^{3}H]PIA and [^{3}H]CGS21680, respectively. [b] Concentrations giving maximal and threshhold stimulation, within the range of 1−1000 nM unless noted, and the percent ± SD of control rate of chloride efflux for each are shown. [c] X = O in the structure above, unless noted. [d] 1-Methyl-3-isobutyl. [e] CPX. [f] 1-Propyl-3-H analogue (Müller et al., 1993). [g] 2-Thio instead of oxo. [h] KW-3902. [i] CSC. [j] XAC. [k] DAX.

The subsequent flux medium consisted of 150 mM sodium gluconate, 1.5 mM potassium gluconate, 10 mM Na-HEPES, pH 7.4, 100 μM bumetanide to inhibit possible efflux via the cotransporter, and different concentrations of CPX or other compounds, as specified. Although the bicarbonate-free condition previously employed (Eidelman et al., 1992) was not found to be necessary, it was sustained in the present experiments. Efflux assays were then performed exactly as described previously (Eidelman et al., 1992), except that the temperature was 19 °C rather than 21 °C. 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.

$^{36}Cl^-$ 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 were calculated by linear regression of the 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 represent the SEM values of four experiments (i.e., $n = 4$), or at least 16 experimental determinations in total.

Northern Blot Analysis. Total cellular RNA was isolated from CFPAC cells using the guanidinium thiocyanate method (Sambrook et al., 1989). Poly(A)+ RNA was selected by two passages over an oligo(dT)−cellulose column (Aviv, 1972). CFPAC and human brain (Clontech) poly(A)+ RNA (7.5 mg) were electrophoresed on a 1% agarose formaldehyde gel (Sambrook et al., 1989), transferred to Hybond-N

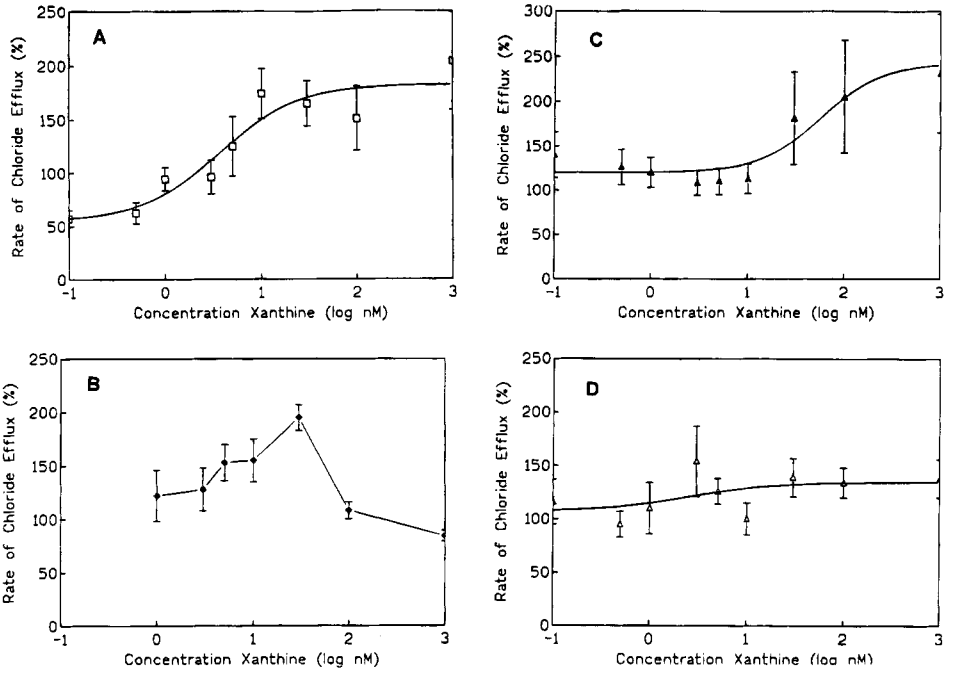


FIGURE 1: Action of CPX on the rate of $^{36}Cl^-$ efflux from pancreatic CFPAC-1 cells, which naturally express CFTR(ΔF508). Panels are data for (A) caffeine, **2**; (B) CPX, **5**; (C) 1,3-diallyl-8-cyclohexylxanthine, **17**; and (D) 8-(cyclohexylmethyl)caffeine, **22**. Cells were pretreated with the indicated concentrations of the xanthine analogue for 15 min at 19 °C and quickly washed, and the efflux of $^{36}Cl^-$ was followed for the subsequent 6 min. The concentration of xanthine was maintained at the indicated concentration throughout the flux experiments. 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 for separate, independent experiments ($n = 4$).

(Amersham), and hybridized in 5× SSPE (0.9 M NaCl, 50 mM $NaPO_4$, pH 7.7, and 5 mM EDTA), 5× Denhardt's solution, 0.5% SDS, 50 mg/mL sonicated salmon testis DNA, and 50% formamide for the CFPAC A_1 blot or 30% formamide for the brain A_1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blots at 42 °C. The human A_1 adenosine receptor probe, corresponding to nucleotides 512−1614 of Accession Number X68485, was randomly primed (Feinberg & Vogelstein, 1983) with [α-^{32}P]dCTP. The GAPDH probe corresponded to nucleotides 475−549 of the rat cDNA (Tso et al., 1985), GenBank Accession Number M17701. Oligonucleotide pairs (overlapping 45-mers) were annealed and filled in with Klenow enzyme and [α-^{32}P]dCTP. Filters were washed in 0.1× SSC at 65 °C and 0.2× SSC at 60 °C for the A_1 and GAPDH probes, respectively.

RESULTS

The structures of compounds **1**−**27** and the affinities in radioligand binding assays at rat brain A_1 and A_{2a} receptors and chloride efflux activities in CFPAC cells are given in Table 1. All of the compounds tested are xanthine derivatives, except for the N^9-alkyladenine derivative **27**, which was chosen as representative of a class of A_1-selective, non-xanthine adenosine antagonists (Thompson et al., 1991). Among the compounds tested are a number of new xanthine derivatives, compounds **15**−**17** and **21**−**24**, substituted at the 1-, 3-, 7-, and 8-positions, which were synthesized by standard methods (Shamim et al., 1989; Shimada et al., 1992; Jacobson et al., 1985, 1993a). At the xanthine 1- and 3-positions, methyl, allyl, or propyl substituents were present. The 7-position substitution in many cases was methyl, but larger groups such as cyclopropylmethyl, **16**, were also included. Several divergent classes of xanthines are represented: mainly 8-cycloalkyl derivatives but also an 8-phenyl derivative, **26**, and an 8-styryl derivative, **25**. In addition several derivatives, **21** and **22**, in which a cycloalkyl group is separated from the xanthine ring system through a methylene group are included.

The naturally occurring xanthines theophylline, **1**, and caffeine, **2**, are weak, nonselective adenosine receptor antagonists and have phosphodiesterase (PDE) inhibitory effects, as well (Jacobson et al., 1992). 3-Isobutyl-1-methylxanthine, **3**, is a more potent PDE inhibitor than caffeine but retains adenosine antagonist properties. Theophylline, **1**, and caffeine, **2** (Figure 1A), at very low concentrations (1−100 nM) were mild stimulants of chloride efflux. These concentrations are well below those needed for adenosine antagonism (10^{-5} M range), suggesting a mechanism for stimulation of chloride efflux other than adenosine antagonism.

Many xanthines, such as **4** and **5**, that are selective for A_1 receptors have been characterized (Shamim et al., 1989; Shimada et al., 1992; Jacobson et al., 1985). CPX, **5**, was the first xanthine reported to stimulate chloride efflux in CFPAC cells. CPX activates the rate of $^{36}Cl^-$ efflux from CFPAC cells in a dose-dependent manner (Figure 1B), having a maximal effect at approximately 30 nM and an ED_{50} of approximately 10 nM. The extent of this increase in efflux over the control level is approximately 200%. Above this concentration (e.g., 1 μM), efflux gradually declines toward the control levels but then rises again at yet higher concentrations (e.g., 10 μM). The high concentration effect has been confirmed by Haws et al. (1994). Since CPX is a highly A_1-selective adenosine antagonist (Table 1), it was presumed that this was the basis for its activity in CFPAC cells (Eidelman, 1992). Consistent with this initial hypoth-

esis, compounds **16** and **22** (Figure 1D), both very weak adenosine antagonists, were nearly inactive in stimulating chloride efflux.

However, methylation of CPX (Shamim et al., 1989) resulted in compound **6**, which had greatly reduced affinity at both A_1 and A_{2a} receptors yet was still moderately active in stimulating chloride efflux, thus contradicting the initial hypothesis. Other A_1-selective antagonists that were active in stimulating chloride efflux were **13**, 8-cyclohexyltheophylline; **17**, 1,3-diallyl-8-cyclohexylxanthine (DAX); **20**, 1,3-dipropyl-8-(cyclohexen-3-yl)xanthine; and **23**, 8-cycloheptyltheophylline. Compound **17** was the most efficacious of all of the analogues examined in stimulating chloride efflux, with levels reaching 260% of control (Figure 1C), although the stimulation occurred at higher concentrations than with CPX. Although the lower potency of **17** (ED_{50} approximately 60 nM) could be seen as consistent with its lower A_1 affinity relative to CPX, many other findings in this study point to the fact that stimulation of chloride efflux is not a result of blocking either A_1 or A_{2a} adenosine receptors. Also, the activity of compound **17** was maintained at concentrations >1 μM.

Compound **11**, a dehydro analogue of CPX that is also highly A_1-selective although less potent (Jacobson et al., 1988), illustrates the divergence of these two activities of xanthine analogues. Compound **11**, at concentrations at which it would block A_1 receptors selectively (10−100 nM), was inactive in stimulating chloride efflux in CFPAC cells.

The ring size of the 8-cycloalkyl group was varied from five to seven carbons. 8-Cyclohexyltheophylline, **13**, displayed weak activity in stimulating chloride efflux. There was some flexibility in the structure−activity relationships (SAR) for chloride efflux elicited by 8-cyclohexylxanthines, although the maximal effect was weak. For example, compound **19**, a 1,3,7-trialkylxanthine, was slightly active. Compound **17** (1,3-diallyl-8-cyclohexylxanthine) was highly efficacious (see above). Increasing the ring size by one methylene group, i.e., 8-cycloheptyltheophylline, **23**, abolished activity in stimulating chloride efflux. 8-Cyclopentyltheophylline (CPT), **4**, at concentrations <1 μM was also inactive, in contrast to the corresponding 1,3-dipropyl analogue CPX, **5**. Thus, the preference for size of the 8-cycloalkyl substituents at this yet-unidentified site of action in CFPAC cells is closely interrelated with other sites on the xanthine molecule.

This set of analogues now permits a systematic analysis of the effects on adenosine receptor affinities by varying the ring size of the 8-cycloalkyl derivatives from four to seven. Among derivatives of theophylline (1,3-dimethylxanthine), the cyclopentyl analogue, **4**, was most A_1-selective (Figure 2A). Among derivatives of caffeine (1,3,7-trimethylxanthine), the cycloheptyl analogue, **24**, showed 6-fold A_{2a} selectivity (Figure 2B), whereas smaller ring sizes (4−6) were nonselective (Jacobson et al., 1993b). These structure−activity relationships diverge greatly from those observed for the same compounds in stimulating Cl^- efflux.

Among other potent A_1-selective antagonists that were similarly inactive or only weakly active in stimulating chloride efflux were analogues closely related to CPX, **5**. Compound **8**, 1,3-dipropyl-8-cyclopentyl-2-thioxanthine (Jacobson et al., 1989), was inactive in CFPAC cells, suggesting that the 2-oxo group is required. Compounds **9**−**11** (Jacobson et al., 1988) contain minor (halogen substitution or

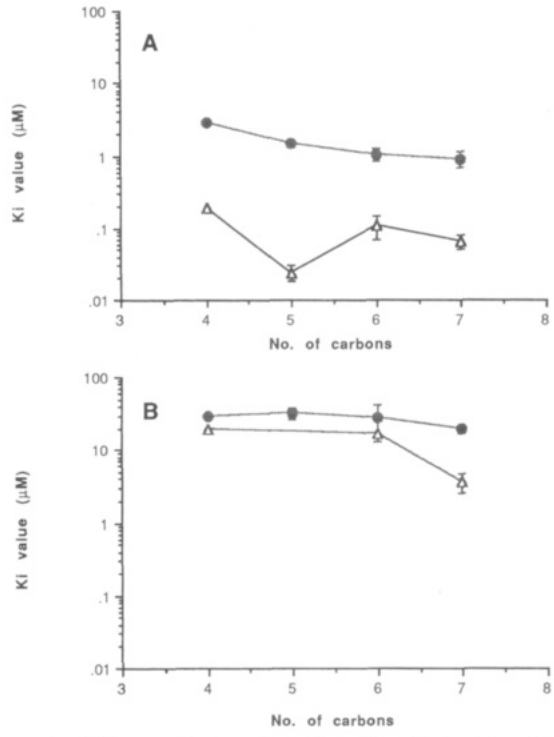


FIGURE 2: Effects of changing ring size of the 8-cycloalkyl substituent on binding at adenosine receptors. Values for rat cortical A_1 receptors (triangles) and rat striatal A_{2a} receptors (circles) are given. Affinity at A_1 and A_2 receptors of (**A**) 8-cycloalkyltheophylline derivatives and of (**B**) 8-cycloalkylcaffeine derivatives is shown. Binding in rat brain was carried out versus 1.0 nM [^{3}H]-PIA at A_1 receptors and versus 5.0 nM [^{3}H]CGS 21680 at A_{2a} receptors. Values are given as the average of two or three (±SEM) determinations performed in triplicate. Data for 8-cyclobutyl derivatives are from Jacobson et al. (1993b).

unsaturation) modifications of the 8-cyclopentyl substituent of CPX and are also inactive in CFPAC cells. This further emphasizes the sensitivity of this effect to structural modifications in the 8-cycloalkyl region, particularly for 5-carbon rings. Compound **12** (Shimada et al., 1992) contains a bulky modification (8-bicyclo group) of CPX and is moderately active in stimulating chloride efflux. Unsaturation introduced in the cyclohexyl ring, i.e., in **20**, was tolerated in the chloride efflux assay. When the cyclohexyl ring was removed from the 8-position by one methylene group (cf. **13** vs **21**), the activity in stimulating chloride efflux diminished, as did the potency as an adenosine antagonist.

XAC, **26**, an 8-phenylxanthine that is highly potent and moderately selective for rat brain A_1 receptors (Jacobson et al., 1985), was relatively inactive in this study in stimulating Cl^- efflux.

Modification of the 1- and 3-substituents, which is known to be a major determinant of potency (van Galen et al., 1992) in binding to A_1 receptors (Pr > Me > allyl for 8-substituted xanthines), did not have a consistent effect on the chloride efflux activity of 8-cycloalkylxanthines. As stated above, a 1,3-dipropyl analog, **5**, was very active, and the corresponding 1,3-dimethyl analog, **4**, was inactive. Among 8-cyclohexylxanthines, a 1,3-dipropyl analog, **18**, was nearly inactive in stimulation of chloride efflux. The corresponding 1,3-dimethyl analogue **13** was weakly active, while 1,3-diallyl, **17**, was highly efficacious at high concentrations.

A recently reported, highly A_{2a}-selective antagonist (Jacobson et al., 1993a), 8-(3-chlorostyryl)caffeine, **25**, was also

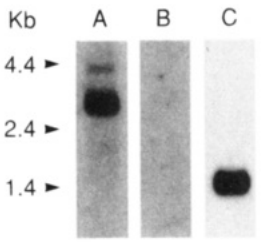


FIGURE 3: Northern blot analysis of human brain and CFPAC cells. (**A**) Human brain and (**B**) CFPAC cell poly(A)+ RNA hybridized with the human A1 adenosine receptor probe (11 day exposure). (**C**) Same blot as in panel B, stripped and hybridized with the GAPDH probe (1-h exposure). Blots were transferred, hybridized and exposed separately.

included in this study. This compound was nearly inactive in stimulating chloride efflux at concentrations comparable to its K_i value at rat striatal A_{2a} receptors (54 nM). The weak activity of **25** and the activity of a number of A_1-selective agonists suggested that blockade of A_{2a} receptors was not the mechanism of stimulation of Cl^- efflux.

Alkyl substitution at the 7-position did not preclude stimulation of chloride efflux. Compound **14**, 8-cyclohexylcaffeine (Shamim et al., 1989), and the corresponding 7-H analogue, **13**, were weakly efficacious in CFPAC cells at concentrations clearly lower than their K_i values at A_1 and A_{2a} receptors. Substitution at the 7-position, especially by Me, is generally thought to decrease potency at A_1 receptors (Shamim et al. 1989); however, compounds **15** (7-ethyl) and **16** (7-cyclopropylmethyl) were somewhat more potent at adenosine receptors than the 7-H analogue. Compounds **15** and **16** were inactive in stimulating chloride efflux.

Lack of expression of A_1 receptors in CFPAC cells was demonstrated by Northern blot analysis (Figure 3). Poly(A)+ RNA isolated from CFPAC cells used in this study showed no hybridization to a human A_1 receptor cDNA probe, excluding this receptor as a mediator of CPX-elicited Cl^- efflux.

DISCUSSION

In this study we have found a number of xanthines that stimulate chloride efflux in CFPAC cells and have demonstrated that there exist distinct patterns in the structure–activity relationships. These patterns do not resemble any of the known subtypes of adenosine receptors (van Galen et al., 1992). The action cannot be through A_1 receptors, since a number of potent and/or selective A_1 antagonists (e.g., **11**, **26**) were inactive. A number of the xanthines that are very potent at A_{2a} receptors (e.g., XAC, **26**), were inactive in stimulating chloride efflux. A potent and selective A_{2a} receptor antagonist, **25**, was only weakly active; thus the stimulation of chloride efflux cannot be through A_{2a} receptors, also as witnessed by the potency of CPX. Several xanthines that were weak antagonists at both A_1 and A_{2a} receptors (such as caffeine) were at least weakly active in the stimulation of chloride efflux. XAC, **26**, is also very potent at A_{2b} receptors with a K_i value of 49 nM (Jacobson et al., 1985), and thus neither A_{2a} nor A_{2b} receptor blockade is a plausible mechanism for the stimulation chloride efflux. Moreover, the most recently defined adenosine receptor subtype, A_3 receptors, does not accommodate most xanthines in the binding site (van Galen et al., 1992; Kim et al., 1994). Finally, although species differences in the affinity of xanthines for adenosine receptors have been noted (Ukena et al., 1986), this is not sufficient to explain the lack of correlation with the activity of these xanthine derivatives in CFPAC cells. Thus, this action of xanthines in stimulating Cl^- efflux from CFPAC cells represents a novel site of action apparently unrelated to any of the known adenosine receptors.

The loss of the effect on stimulation of chloride efflux at high concentrations of the drug (e.g., Figure 1A) is yet unexplained but may be related to nonselectivity at these concentrations. However, this multiphasic behavior as seen with **5** is not general for all of the xanthine derivatives and so appears to be an idiosyncratic property of only some of the compounds.

Does this action represent a new subtype of adenosine receptors or a yet-to-be-characterized xanthine binding site common to both human (e.g., CFTR) and mouse cells [e.g., 3T3 cells; see Guay-Broder et al. (1995)]? It would be helpful to characterize the action of adenosine agonists at this site as well as adenosine antagonists, since in the original study adenosine deaminase had an effect in the same direction as the effect of CPX. Further exploration of this point would distinguish a new adenosine receptor from a xanthine (non-adenosine) binding site. An alternative mechanistic possibility includes direct action of CPX upon the ΔF508 mutant of CFTR. This could be tested by attempted binding of [^{3}H]CPX to purified CFTR or CFTR(ΔF508) or by the development of affinity labels based on the present SAR patterns. In fact, CPX action is noted only in cells bearing CFTR(ΔF508) and not in cells lacking CFTR altogether (e.g. NIH 3T3 cells) or cells bearing wild-type CFTR (e.g., T84 cells, NIH 3T3, CFTR).

The CFPAC cell line was derived from pancreatic tissue of a CF patient homozygous for ΔF508, and we have therefore used potency in this assay as potentially predictive for use of the active xanthines for treatment of CF. CPX has already been proposed as a candidate drug to treat the chloride permeability defect associated with cystic fibrosis and thus warranted the present substantial expansion of the structure–activity relationships using a collection of 26 analogues. As a result we know that it is possible to achieve the same effect on chloride efflux as with CPX but without the mediation of presently known adenosine receptors. This may have important implications for potential drug therapy for CF. For example, using a xanthine that is a weak adenosine antagonist or not an antagonist at all would diminish the potential of cardiac, central nervous system, or other side effects potentially associated with adenosine antagonism (Jacobson et al., 1991). For example, compounds **17**, 1,3-diallyl-7-cyclohexylxanthine; **19**, 1,3-dipropyl-7-methylcyclohexylxanthine; and **14**, cyclohexylcaffeine, have these properties and may serve as lead compounds for future drug development.

In conclusion, in an effort to identify potential therapeutic agents for cystic fibrosis, we have tested a variety of xanthine derivatives in the novel action as stimulators of chloride efflux. We have used as a model a transformed cell line derived originally from a cystic fibrosis patient and containing the most common genetic defect of the chloride transporter. A comparison of chloride effects with the structure–activity relationships of xanthines at all of the known adenosine receptors has revealed no correlation, suggesting a novel mechanism, perhaps involving a direct interaction of the xanthine with the CFTR protein.

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