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Structural basis for specificity and potency of xanthine derivatives as activators of the CFTR chloride channel

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1 On the basis of their structure, we compared the ability of 35 xanthine derivatives to activate the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel stably expressed in chinese hamster ovary (CHO) cells using the cell-attached patch clamp and iodide efflux techniques.

2 Activation of CFTR channels was obtained with 3-mono, 1,3-di or 1,3,7-tri-substituted alkyl xanthine derivatives (enprofylline, theophylline, aminophylline, IBMX, DPMX and pentoxifylline). By contrast, xanthine derivatives substituted at the C8- or N9-position failed to open CFTR channels.

3 The CFTR chloride channel activity was blocked by glibenclamide (100 μM) but not by DIDS (100 μM).

4 Activation of CFTR by xanthines was not mimicked by the calcium ionophore A23187, adenosine, UTP, ATP or the specific phosphodiesterase inhibitors rolipram, Ro 20-1724 and milrinone. In addition, we found no correlation between the effect of xanthines on CFTR and on the cellular cyclic AMP or ATP levels.

5 We then synthesized a series of 3,7-dimethyl-1-alkyl xanthine derivatives; among them, 3,7-dimethyl-1-propyl xanthine and 3,7-dimethyl-1-isobutyl xanthine both activated CFTR channels without increasing the intracellular cyclic AMP level, while the structurally related 3,7-dimethyl-1-(2-propenyl) xanthine and 3,7-dimethyl-1-(oxiranyl methyl) xanthine were inactive.

6 Our findings delineate a novel function for xanthine compounds and identify the molecular features that enable xanthine activation of CFTR. These results may be useful in the development of new molecules for studying the pharmacology of chloride channels.

Keywords: CFTR chloride channel; xanthine derivatives; iodide efflux; patch clamp

Introduction

The importance and diversity of chloride channels in cell membranes are widely recognized, particularly now that defective chloride channels are known to underlie cystic fibrosis (CF) (Riordan *et al*., 1989; Tsui & Buchwald, 1991), Dent's disease (Steinmeyer *et al*., 1995) and myotonia (Koch *et al*., 1992). Despite our growing knowledge about their molecular structures, biophysical properties and regulation, their pharmacologies remain poorly developed and few specific openers and blockers of chloride channels have been identified so far.

Cystic fibrosis is characterized by defective adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent chloride conductance in epithelial cells and is caused by a defect in the targeting of the chloride channel CFTR (CF transmembrane conductance regulator) to the plasma membrane, and to channel dysfunction at the cell surface (Riordan *et al*., 1989; Tsui & Buchwald, 1991; Dalemans *et al*., 1991; Hanrahan *et al*., 1995). In the presence of forskolin, the methylxanthine drug IBMX (1-methyl-3-isobutyl xanthine) has been shown to activate normal and mutated CFTR chloride channels in epithelia (Hanrahan *et al*., 1995), *Xenopus* oocytes (Drumm *et al*., 1991) and transfected cells (Becq *et al*., 1994). Activation of CFTR channels with IBMX or theophylline in the absence of forskolin has also been demonstrated in human pancreatic duct (Becq *et al*., 1993a) and tracheo-bronchial gland cells (Becq *et al*., 1993b). These observations were initially attributed to inhibition by xanthines of phosphodiesterase (PDE) activity, which would elevate cyclic AMP by inhibiting its degradation. However, other mechanisms are likely to contribute to the activation of CFTR. In particular, substituted xanthines inhibit alkaline phosphatase (Croce *et al*., 1979; Becq *et al*., 1993a) and antagonize adenosine receptors A (Bruns *et al*., 1983). Using CF pancreatic duct cells, Eidelman *et al*. (1992) have shown that chloride current can be elicited by CPX (1,3-dimethyl-8-cyclopentyl xanthine) a potent A_1 adenosine-receptor antagonist.

The role of CFTR in the chloride conductance of several tissues (e.g. in the heart, nephron or exocrine pancreas) may be clarified by the use of specific activators. The main goal of this study was to design openers of CFTR channels from the xanthine family. We have studied the effect on CFTR of chemical modifications of the xanthine skeleton (see Figure 1a) using 35 alkyl-substituted xanthine derivatives. The results show a correlation between the potency of a series of 1,3,7 trialkyl xanthine derivatives and the opening of the CFTR chloride channel. Moreover, xanthines substituted at the nitrogen N9 or at the carbon C8 failed to open CFTR, indicating that the mechanism of CFTR opening by xanthines required both 1,3,7-trialkyl substitution and non-substituted C8- and N9-positions. We also showed that both active and inactive forms of a given CFTR channel opener could be synthesized by manipulating the nature of the chemical group

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at N1. Some of these results have been published in a preliminary form (Becq *et al*., 1996a).

Methods

Chemicals and solutions

Tables 1, 2 and 3 present the xanthine derivatives used in this study. Compounds X-02, 03, 04, 05, 07, 08, 09, 12, 15, 16, 20, 22, 23, 24, 25, 26 and 35 were from Research Biochemicals International (RBI, Natick, MA, U.S.A.). Compounds X-01, 06, 10, 11, 13, 14, 17, 18, 19 and 21 were from Sigma (St Louis, MO). Forskolin, milrinone and 8-methoxymethyl IBMX were from Calbiochem. Rolipram and Ro 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone) were from RBI. Other chemicals were from Sigma. All xanthine derivatives were prepared freshly before the experiment and dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the experiment was less than 0.1% and was found to have no significant effect on iodide efflux or membrane currents.

Cell culture

Chinese hamster ovary (CHO-K1) cells stably transfected with either pNUT vector alone (CFTR (−) CHO cells) or pNUT containing wild type CFTR (CFTR(+) CHO cells) were provided by J.R. Riordan and X.-B. Chang, Scottsdale, AZ, U.S.A. (Tabcharani *et al*., 1991; Chang *et al*., 1993). Cells cultured at 37°C in 5% CO_2 were maintained in αMEM containing 7% foetal bovine serum, antibiotics (50 iu penicillin ml^{-1} and 50 $\mu g\ ml^{-1}$ streptomycin) and 100 μM methotrexate (all from Sigma, St Louis, MO). Detailed procedures for culture and transfection appeared elsewhere (Tabcharani *et al*., 1991; Chang *et al*., 1993; Becq *et al*., 1994).

Isotopic iodide efflux

CFTR chloride channel activity was assayed by measuring iodide (^{125}I) efflux from transfected CHO cells as described previously (Becq *et al*., 1996b). All experiments were performed at 37°C. CHO cells grown for four days in 12-well plates were washed twice with 2 ml of modified Earle's salt solution (solution B) containing (in mM): NaCl 137, KCl 5.36, $MgCl_2$ 0.8, $CaCl_2$ 1.8, glucose 5.5 and HEPES 10, pH 7.4. Cells were then incubated in B medium containing 1 μM KI (1 μCi $Na^{125}I\ ml^{-1}$, NEN, Boston, MA) for 30 min at 37°C to permit the iodide to reach equilibrium. Cells were washed three times with 1 ml of B medium. After one min, the B medium was removed to be counted and quickly replaced by 1 ml of the same medium. This procedure was repeated every min for 11 min. The first two aliquots were used to establish a stable base line in efflux buffer alone. B medium containing the appropriate drug was used for the remaining aliquots. At the end of the incubation, the medium was collected and cells were solubilized in 1 N NaOH. The radioactivity was determined by use of a gamma counter (Kontron). The total amount of ^{125}I (in c.p.m.) at time 0 was calculated as the sum of c.p.m. counted in each 1 min sample plus the c.p.m. in the NaOH fraction. Efflux curves were constructed by plotting the % of cellular content accumulated in the medium versus time. The cumulative efflux at 5 min (noted T5) was further used to compare the efficiency of drug with the control. Data are presented as the mean ± s.d. of *n* separate experiments. Statistics were determined by use of the Statgraphics software with two-sample analysis and *t* test. $*P<0.05$; $**P<0.01$; $***P<0.001$.

Single-channel patch-clamp recording

CHO cells were plated on 35 mm Petri dishes and cultured at 37°C in 5% CO_2 for 1–4 days before use. Single-channel currents were recorded from cell-attached patches. Cells were stimulated with forskolin or appropriate xanthines at the concentrations indicated in the text (dissolved in dimethyl sulphoxide DMSO; final DMSO concentration: 0.1%). Experiments were performed at room temperature. Results were displayed conventionally with inward currents (outward flow of anions) indicated by downward deflections. In all the figures, dashed lines give the zero current baselines when the channels were in the closed state. Potentials were expressed as the bath potential minus the patch electrode potential. The pipette solution contained (in mM): NaCl 150, $MgCl_2$ 2 and TES 10 (pH 7.4); the bath contained (in mM): NaCl 145, KCl 4, $MgCl_2$ 2 and TES 10 (pH 7.4). Other details appeared elsewhere (Becq *et al*., 1993b). Data are presented as the mean ± s.d. of *n* separate experiments.

Measurement of cellular cyclic AMP

CHO cells grown for four days in 12-well plates were washed twice with 2 ml of B medium. 0.5 ml of B medium containing the test compound was added to each well. After a 5 min incubation period at 37°C, the reaction was stopped by adding 55 μl of 11 N perchloric acid. Cells were frozen for later cyclic AMP determination by use of a radioimmuno assay kit (RIANEN kit, NEN). Cyclic AMP levels were expressed as pmol/well.

Measurement of cellular ATP

ATP was measured with the luciferin-luciferase method. A bioluminescent kit (CLS Test Combination from Boehringer, Mannheim, GmbH) was used for determination (in triplicate) of cellular ATP. In order to compare the effect of different drugs, test data are expressed as % of ATP content of cells incubated in the absence of drugs.

General procedure for the synthesis of compounds X-27 to X-34

Our procedure for the synthesis of xanthines was adapted from Cottam *et al*. (1996). The starting material was IBMX (1.13 g, 5 mM) for compounds X-27 to X-29; xanthine (0.76 g, 5 mM) for X-30; and theobromine (0.9 g, 5 mM) for X-31 to X-34. The respective solution in dry dimethylformamide (DMF, 75 ml) was stirred under nitrogen before the addition of NaH (5.5 mM). The mixture was then brought to 75°C for 30 min before the addition of the appropriate alkylhalide (5.5 mM). After 4–10 h at 75°C, the reaction mixture was allowed to cool and then 1 ml H_2O was added. The solvent and H_2O were removed *in vacuo*, and the dry residue was extracted with methylene chloride. The solution was dried over Na_2SO_4 and chromatographed on silica gel and eluted with chloroform or ethyl acetate. The product was purified further by sublimation. ^1H n.m.r. spectra, IR spectra and elemental analyses were consistent with the assigned structures. X-27: 1,7-dimethyl-3-isobutyl xanthine; white powder, 1.04 g (88%), melting point (mp) 99–100°C; X-28: 1-methyl-7-propyl-3-isobutyl xanthine; white powder, 1.16 g (88%), mp 102–103°C; X-29: 1-methyl-3-isobutyl-7-cyclopentyl xanthine; white powder, 0.91 g

(63%), mp 93°C; X-30: 1,3,7-triisobutyl xanthine; white powder, 0.09 g (6%), mp 81°C; X-31: 3,7-dimethyl-1-(oxiranyl methyl) xanthine; white powder, 0.55 g (47%), mp 140°C; X-32: 3,7-dimethyl-1-propyl xanthine; white powder, 1.04 g (94%); mp 138°C (Daly *et al*., 1986); X-33: 3,7-dimethyl-1-isobutyl xanthine; white powder, 0.75 g (63%); mp 145°C; X-34: 3,7-dimethyl-1-(2-propenyl) xanthine); white powder, 0.75 g (68%); mp 143°C (Daly *et al*., 1986).

Results

Stimulation of CFTR channels by xanthine derivatives

We have previously characterized the activation of CFTR chloride channels by cyclic AMP agonists using CHO cells stably expressing the CFTR protein and the iodide efflux technique (Hanrahan *et al*., 1995; Becq *et al*., 1996b). Basal effluxes (cumulative effluxes at 5 min, noted hereafter T5 in %) from CFTR(−) and CFTR(+) CHO cells were $44\pm7\%$ ($n=6$) and $49\pm5\%$ ($n=32$, Table 1) of the initial cellular content, respectively. No calcium-dependent chloride conductance was detected in these cells by use of A23187 (5 μM, $n=4$) or ionomycin (5 μM, $n=4$) as calcium agonists (not shown). In the presence of adenosine (100 μM) the cumulative efflux ($T5=48\pm1\%$, $n=4$) was not statistically different from the control value ($49\pm5\%$, $n=32$). As expected, the iodide efflux was significantly increased ($P<0.001$) by forskolin (5 μM) only in the CFTR(+) CHO cells (CFTR(−) CHO, $T5=47\pm1\%$, $n=4$; CFTR(+) CHO, $T5=88\pm4\%$, $n=14$).

We then examined the effect of 35 substituted xanthines on the activation of CFTR chloride channels with CFTR(+) CHO cells. Tables 1–3 show the chemical structure of the xanthine derivatives tested (see Figure 1a for nomenclature of N and C atoms) and the cumulative efflux measured 5 min after stimulation with 500 μM of the xanthine of interest.

Xanthine itself (X-01) did not activate CFTR channel (Figure 1b and Table 1). Compounds having a single methyl group at either N1, N3, N7 or N9 also failed to activate CFTR (X-02 to X-05, Table 1). Activation of CFTR channels was obtained with dimethyl xanthines but this depended on the position of the substituents in the xanthine skeleton. When methyl groups were placed at N9 and either N1, N3 or N7 (X-07 to X-09) the compound had no effect (Table 1). By contrast,

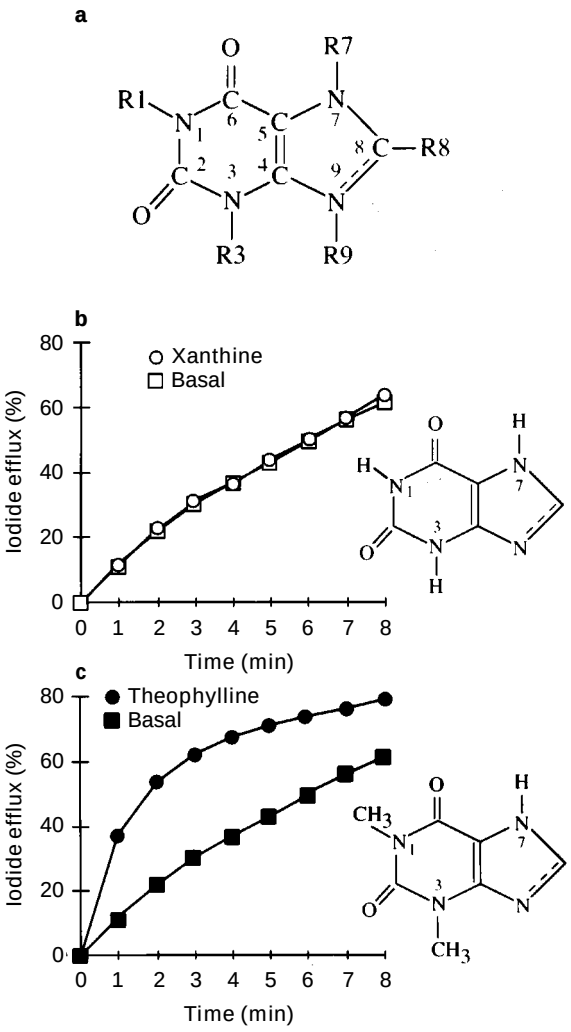

Figure 1 Comparison of xanthine- and theophylline-stimulated iodide efflux. (a) Chemical structure of xanthine showing the possible substitutable positions N1, N3, N7, C8 and N9. (b) and (c) ^{125}I efflux from CFTR(+) CHO cells in the presence or absence (basal) of 500 μM xanthine (b) or theophylline (c). Effluxes are expressed as % of cellular content as described in the methods section and are noted iodide efflux on vertical axis. The compounds were added at time zero. The chemical structure of the compounds is given.

Table 1 Structure of methylxanthine derivatives and their effect on the activation of CFTR chloride channels

No.	*Chemical structure*	*R1*	*R3*	*R7*	*R8*	*R9*	*Iodide efflux*	n	t-*test*
Basal							49±5	32	
Forskolin							88±4	14	***
X-01	Xanthine	−H	−H	−H	−H	−H	48±4	4	NS
X-02	1-Methylxanthine	$-CH_3$	−H	−H	−H	−H	48±2	4	NS
X-03	3-Methylxanthine	−H	$-CH_3$	−H	−H	−H	47±4	5	NS
X-04	7-Methylxanthine	−H	−H	$-CH_3$	−H	−H	49±3	4	NS
X-05	9-Methylxanthine	−H	−H	−H	−H	$-CH_3$	51±2	4	NS
X-06	1,3-Dimethylxanthine	$-CH_3$	$-CH_3$	−H	−H	−H	68±6	8	**
X-07	1,9-Dimethylxanthine	$-CH_3$	−H	−H	−H	$-CH_3$	50±9	2	NS
X-08	3,9-Dimethylxanthine	−H	$-CH_3$	−H	−H	$-CH_3$	53±2	4	NS
X-09	7,9-Dimethylxanthine	−H	−H	$-CH_3$	−H	$-CH_3$	45±1	4	NS
X-10	3,7-Dimethylxanthine	−H	$-CH_3$	$-CH_3$	−H	−H	51±2	4	NS
X-11	1,3,7-Trimethylxanthine	$-CH_3$	$-CH_3$	$-CH_3$	−H	−H	56±6	6	**
X-12	1,3,9-Trimethylxanthine	$-CH_3$	$-CH_3$	−H	−H	$-CH_3$	46±3	4	NS

The activity of CFTR channels was evaluated by use of the iodide efflux method. Experiments were performed with CFTR(+) CHO cells. For each compound tested, the iodide efflux after 5 min (T5%±s.d.) is presented with the number of experiments. *t* test: ** $P<0.01$; *** $P<0.001$; NS, no significant differences. Statistical differences are given compared to the basal iodide efflux (no compound added, $n=32$). The iodide efflux in the presence of 5 μM forskolin is given for comparison ($n=14$).

drugs with methyl groups at N3 and N1, theophylline (1,3-dimethyl xanthine, X-06, Figure 1c and Table 1) or aminophylline (theophylline ethylenediamine, T5 = 59 ± 5%, $n = 6$, $P < 0.01$) activated CFTR. Finally, the trimethylation at N1, N3 and N7 (caffeine, X-11) or N1, N3 and N9 (isocaffeine, X-12) had a minor effect (Table 1). Therefore among the methyl xanthine derivatives tested, only compounds doubly substituted by methyl at N1 and N3 were able to activate CFTR. Xanthines substituted by a single methyl or by three methyl groups had no effect.

In the following experiments, we analysed the effect on CFTR of alkyl substitutions in the xanthine skeleton. Among the compounds tested (Table 2), pentoxifylline bearing a 1-(5-oxo-hexyl) doubly substituted by 1,3-dipropyl (X-24), 3-*n*-propyl xanthine (enprofylline, X-14), IBMX (X-17) and 7-methyl-1,3-dipropylxanthine (DPMX, X-25) activated CFTR (Table 2). Both the time course and magnitude of the iodide efflux in response to IBMX or DPMX were similar to those observed with 5 μM forskolin (see Figure 2a).

Table 2 Structure of alkylxanthine derivatives and their effect on the activation of CFTR chloride channels

No.	*Chemical structure*	*R1*	*R3*	*R7*	*R8*	*R9*	*Iodide efflux*	n	t-*test*
X-14	3-n-Propylxanthine	$-H$	$-(CH_2)_2CH_3$	$-H$	$-H$	$-H$	60 ± 1	6	**
X-15	7-n-Propylxanthine	$-H$	$-H$	$-(CH_2)_2CH_3$	$-H$	$-H$	55 ± 1	4	NS
X-16	9-n-Propylxanthine	$-H$	$-H$	$-H$	$-H$	$-(CH_2)_2CH_3$	51 ± 2	3	NS
X-17	1-Methyl-3-isobutyl-xanthine	$-CH_3$	$-CH_2$-CH $(CH_3)_2$	$-H$	$-H$	$-H$	87 ± 5	21	***
X-18	1,3-Dimethyl-7-(2-carboxyethyl) xanthine	$-CH_3$	$-CH_3$	$-CH_2COOH$	$-H$	$-H$	56 ± 2	4	**
X-19	1,3-Dimethyl-8-phenyl xanthine	$-CH_3$	$-CH_3$	$-H$	$-C_6H_5$	$-H$	55 ± 6	4	NS
X-20	1,3-Diethyl-8-phenyl xanthine	$-CH_2CH_3$	$-CH_2CH_3$	$-H$	$-C_6H_5$	$-H$	56 ± 5	4	NS
X-21	1,3-Dimethyl-8 (3-carboxypropyl) xanthine	$-CH_3$	$-CH_3$	$-H$	$-(CH_2)_2COOH$	$-H$	48 ± 2	4	NS
X-22	1,3-Dimethyl-8-cyclopentyl xanthine	$-CH_3$	$-CH_3$	$-H$	$-C_5H_9$	$-H$	49 ± 2	4	NS
X-23	1,3-Dipropyl-8-cyclopentyl xanthine	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	$-H$	$-C_5H_9$	$-H$	52 ± 4	4	NS
X-24	3,7-Dimethyl-1-(5-oxo-hexyl) xanthine	$-(CH_2)_4COCH_3$	$-CH_3$	$-CH_3$	$-H$	$-H$	63 ± 4	5	**
X-25	7-Methyl-1,3-dipropyl xanthine	$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	$-CH_3$	$-H$	$-H$	82 ± 5	8	***

Experiments were performed with CFTR(+) CHO cells. The activity of CFTR channels was evaluated by use of the iodide efflux method. Values of iodide efflux are given as described in the legend of Table 1. Iodide efflux after 5 min (T5) is presented % ± s.d. with the number of experiments. *t* test: ** $P < 0.01$; *** $P < 0.001$; NS, no significant differences. Statistical differences are given compared to the basal iodide efflux.

Table 3 Structure of alkylxanthine derivatives and their effect on the activation of CFTR chloride channel

No	*Chemical structure*	*R1*	*R3*	*R7*	*R8*	*Iodide efflux*	*n*	t *test*
X-17	1-Methyl-3-isobutyl xanthine	$-CH_3$	$-CH_2$ $-$CH $(CH_3)_2$	$-H$	$-H$	87 ± 5	21	**
X-26	1-Methyl-8-methoxy-3-isobutyl xanthine	$-CH_3$	$-CH_2$ $-$CH $(CH_3)_2$	$-H$	$-OCH_3$	55 ± 3	4	NS
X-27	1,7-Dimethyl-3-isobutyl xanthine	$-CH_3$	$-CH_2$ $-$CH $(CH_3)_2$	$-CH_3$	$-H$	52 ± 2	4	NS
X-28	1-Methyl-7-propyl-3-isobutyl xanthine	$-CH_3$	$-CH_2$ $-$CH $(CH_3)_2$	$-(CH_2)_2CH_3$	$-H$	50 ± 1	4	NS
X-29	1-Methyl-3-isobutyl -7-cylopentyl xanthine	$-CH_3$	$-CH_2$ $-$CH $(CH_3)_2$	$-C_5H_9$	$-H$	38 ± 5	2	**
X-30	1,3,7 -Triisobutyl xanthine	$-CH_2-CH(CH_3)_2$	$-CH_2$ $-$CH $(CH_3)_2$	$-CH_2-CH(CH_3)_2$	$-H$	41 ± 1	4	**
X-31	3,7-Dimethyl-1-(oxiranyl methyl) xanthine	$CH_2-CH-CH_2$ (O)	$-CH_3$	$-CH_3$	$-H$	51 ± 4	3	NS
X-32	3,7-Dimethyl-1-propyl xanthine	$-(CH_2)_2CH_3$	$-CH_3$	$-CH_3$	$-H$	71 ± 3	6	**
X-33	3,7-Dimethyl-1-isobutyl xanthine	$-CH_2-CH(CH_3)_2$	$-CH_3$	$-CH_3$	$-H$	75 ± 4	6	***
X-34	3,7-Dimethyl-1-(2-propenyl) xanthine	$-CH_2$ $-CH=CH_2$	$-CH_3$	$-CH_3$	$-H$	44 ± 2	6	*
X-35	3,7-Dimethyl-1-propargyl xanthine	$-CH_2C\equiv CH$	$-CH_3$	$-CH_3$	$-H$	53 ± 2	4	NS

Experiments were performed with CFTR(+) CHO cells. The activity of CFTR channels was evaluated by use of the iodide efflux method. Values of iodide efflux are given as described in the legend of Table 1. Iodide efflux after 5 min (T5) is presented % ± s.d. The number of experiments is given. *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, no significant differences. Statistical differences are given compared to the basal iodide efflux.

The stimulation of iodide efflux by forskolin, IBMX or DPMX was concentration-dependent with a half-maximal effect at ≈ 0.5 μM, ≈ 100 μM and ≈ 250 μM, respectively. The stimulation of the iodide efflux by forskolin (5 μM), DPMX (500 μM) or IBMX (500 μM) was reduced to the basal level by 100 μM glibenclamide (Table 4). By contrast, the maximal effect of forskolin, IBMX or DPMX was not affected by 100 μM DIDS (Table 4), indicating that CFTR was indeed the only chloride channel activated by these compounds in CFTR(+) CHO cells. Other chloride channels (high-conductance anion channels and outwardly rectifying chloride channels) endogenously expressed in CHO cells, are both sensitive to DIDS (F. Becq, unpublished single channel data).

To confirm iodide efflux data, we also performed cell-attached patch-clamp experiments to characterize the xanthine-activated channel. In control patch-clamp experiments with CFTR(+) CHO cells, i.e. in the absence of cyclic AMP agonists, no spontaneous channel activity was recorded in the cell-attached configuration ($n=30$). As shown in Figure 2b, addition to the bath of 10 μM forskolin to a previously silent cell-attached patch (Figure 2b) caused progressive opening of multiple CFTR channels within 2 min. The current-voltage relationship and unitary conductance (6.8 ± 0.21 pS, $n=6$, Figure 2e) were similar to those already described for this preparation (Tabcharani *et al*., 1991; Becq *et al*., 1994). Openings of CFTR channels were also

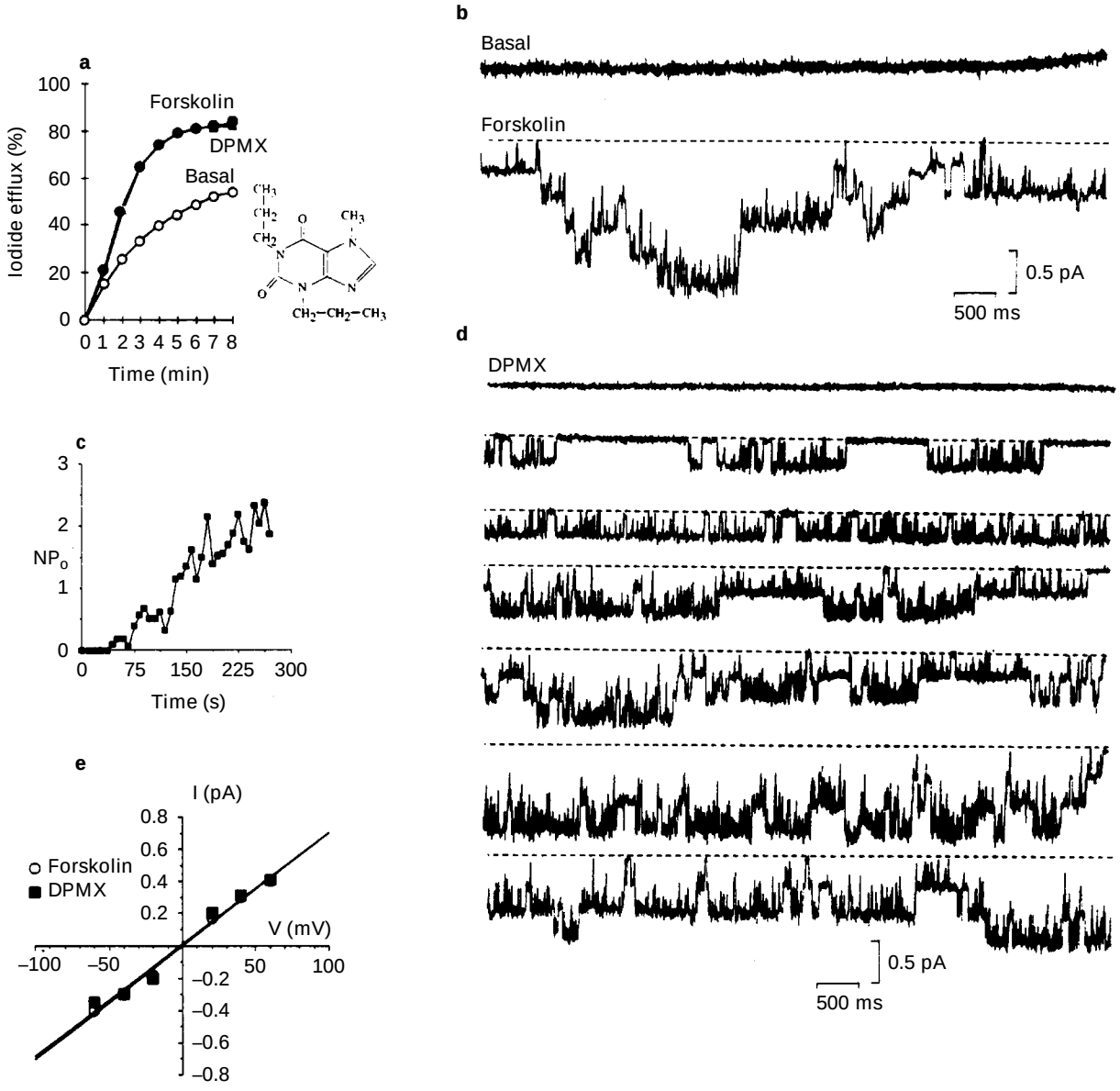

Figure 2 Activation of CFTR channels by 7-methyl-1,3-dipropylxanthine (DPMX). (a) ^{125}I efflux from CFTR(+) CHO cells in resting state (basal) or in the presence of 500 μM DPMX or 5 μM forskolin. The compounds were added at time zero. The chemical structure of DPMX is shown. (b) Representative recording of CFTR channels in a cell-attached patch ($-V_{pipette} = -30$ mV) in the presence of 10 μM forskolin (bottom trace); note the absence of channel activity before drug addition (upper trace). (c) Evolution of NP_o (N: number of channels per patch, P_o: open probability) as a function of time from a cell-attached patch stimulated with 500 μM DPMX. Time zero indicates when DPMX was added. (d) Seven continuous traces showing the progressive activation of CFTR channels in the cell-attached configuration ($-V_{pipette} = -30$ mV) in the presence of 500 μM DPMX (added at the beginning of the first top trace); note the absence of channel activity at the beginning of the recording and the progressive opening of three channels. DPMX was present throughout the recordings. (e) Current-voltage relationship of CFTR channels activated by DPMX and forskolin in the cell-attached configuration as indicated. V(mV) represents $-V_{pipette}$ in mV.

Table 4 Effect of DIDS and glibenclamide on CFTR activity

	Control	*DIDS*	*Glib*
Basal	48±5	32±2	43±7
Forskolin	87±2	87±2.5	54±3
DPMX (X-25)	77±3.5	68±2	45±3
IBMX (X-17)	78±1	75±1	56±2

Experiments were performed by use of the iodide efflux method. CFTR(+) CHO cells were exposed to DIDS (100 μM) or glibenclamide (100 μM) in the presence of forskolin (5 μM), 7-methyl-1,3-dipropyl xanthine (DPMX, 250 μM) or 1-methyl-3-isobutyl xanthine (IBMX, 250 μM). Data are presented as iodide efflux (T5%±s.d.) for four to six separate experiments.

consistently observed following addition of 500 μM DPMX (X-25, $n=7$) to the bath as shown in Figure 2c and d. The current-voltage relationship and unitary conductance (6.9±0.1 pS, $n=7$) of DPMX-stimulated CFTR channels was again similar to that obtained during forskolin stimulation (Figure 2e). Similar activation was observed with IBMX, theophylline, aminophylline, and 3-*n*-propyl xanthine (not shown). By contrast, 1,3,7-trimethyl xanthine (X-11, $n=6$), and 8-substituted xanthine derivatives (X-19, $n=8$; X-20, $n=4$; and X-23, $n=12$) all failed to open CFTR channels in the cell-attached configuration, confirming the efflux data. In CFTR(−) CHO cells, neither forskolin nor xanthines have a detectable effect on either the membrane current or the iodide efflux (not shown).

From the efflux data presented in Tables 1 and 2 and the patch-clamp experiments, it can be concluded that compounds substituted at C8 (X-19 to X-23) or N9 (X-05, X-07, X-08, X-09, X-12 and X-16) failed to open CFTR. Moreover, whereas theophylline opened CFTR, an additional substitution at the C8 (X-19, X-21 and X-22) abolished the potency of the compound (Table 2). A similar observation was made by comparing DPMX (X-25), a potent activator of CFTR, and CPX (X-23), which was not able to open CFTR (Table 2).

Structure-activity relationship of alkylxanthines

On the basis of the above structure-activity relationships, we anticipated that xanthines substituted at N1, N3 and N7 should be able to open CFTR channels. To test this hypothesis we synthesized 1,3,7-trialkylxanthines and evaluated their capability as activator of CFTR. Table 3 shows the chemical structure of 8 synthesized xanthines (X-27 to X-34) compared to IBMX (X-17) and two commercial xanthines (X-26 and X-35). Modification of IBMX either by substitution at C8 (X-26), N7 (X-27, X-28, and X-29), or N1 and N7 (X-30) abolished the potency of the compound (i.e. of IBMX, Table 3).

Then, we decided to stabilize two positions, N3 and N7, with a methyl group and to study the effect of various substitutions at N1. As shown in the Table 3 only X-32 or X-33 stimulated the activity of CFTR. The iodide efflux observed in the presence of these two compounds (Figure 3a and b) was similar to that observed with DPMX (X-25) or forskolin (see Figure 2a). Interestingly, a slight modification of the substituent group at N1 abolished the effect on CFTR (compare the structure of X-31, X-34 and X-35 with that of X-32 or X-33, Table 3). These data indicate that xanthine activation of CFTR depends not only on the position, but also the nature of the substituent, since the presence of a double bond (X-34, Figure 3c), a triple bond (X-35), or an epoxy group (X-31) all resulted in inactive compounds. Although not activators of CFTR, X-29, X-30 and X-34 appeared to inhibit slightly the iodide efflux (Table 3).

The iodide efflux data were also matched by single channel recordings. As shown in Figure 3d and e, openings of CFTR channels in the cell-attached configuration were observed following addition to the bath of 3,7-dimethyl-1-propyl xanthine (X-32, 500 μM, $n=7$) and 3,7-dimethyl-1-isobutyl xanthine (X-33, 500 μM, $n=6$). Again, the current-voltage relationships of X-32- or X-33-stimulated CFTR channels (Figure 3g) were similar to that obtained with forskolin (Figure 2e). Finally, Figure 3f shows that the xanthine derivative 3,7-dimethyl-1-(2-propenyl) xanthine (X-34, 500 μM, $n=4$) failed to open CFTR channels in the cell-attached configuration, as expected from the macroscopic flux experiments shown in the Figure 3c.

Effect of forskolin and xanthines on intracellular cyclic AMP

Because CFTR is a cyclic AMP-dependent chloride channel (for review see Hanrahan *et al*., 1995), we tested the possibility that activation by xanthine might be due to elevation of cyclic AMP. In resting CFTR(+) CHO cells, the cellular cyclic AMP content was 1.4±0.3 pmol cyclic AMP/well ($n=6$). Rolipram (a specific cyclic AMP-dependent phosphodiesterase inhibitor, 100 μM, $n=3$) and forskolin (5 μM, $n=4$) increased the cyclic AMP levels measured after 5 min to 6.2±0.2 pmol cyclic AMP/well and 25±1.7 pmol cyclic AMP/well, respectively (Figure 4a). Forskolin caused a dose-dependent elevation of cyclic AMP in CFTR(+) CHO cells with an $EC_{50} \approx 0.5$ μM (not shown). Both IBMX and rolipram induced a similar rise in the cellular cyclic AMP level after 5 min (IBMX, 500 μM, 7.8±1.0 pmol cyclic AMP/well, $n=4$, Figure 4b). The corresponding cyclic AMP level in the presence of 8-phenyltheophylline, caffeine, pentoxifylline and DPMX ($n=3$ for all compounds at 500 μM) is presented in Figure 4b (4.7±0.2, 2.3±0.9, 4.2±1.2 and 6.8±1.8 pmol cyclic AMP/well, respectively).

We then reasoned that if xanthine derivatives open CFTR channels by inhibiting phosphodiesterase (PDE) activity (i.e. though a cyclic AMP-dependent mechanism), more specific PDE inhibitors should also activate CFTR. However, using both cell-attached patch-clamp recording (not shown) or iodide efflux (Figure 5), we failed to detect any activation of CFTR chloride channels in CFTR(+) CHO cells by the PDE inhibitors rolipram, Ro 20-1724, milrinone and 8-methoxymethyl IBMX. Since activation of CFTR was not mimicked by various specific non-xanthine phosphodiesterase inhibitors, this implies that xanthine derivatives activate CFTR by a cyclic AMP-independent mechanism. This was further confirmed by observing that X-31, X-32, X-33 and X-34 (500 μM) had minimal effects on cellular cyclic AMP levels in CFTR(+) cells (4.2±2, 1.7±0.5, 2.6±0.5, and 2.3±1.6 pmol cyclic AMP/well, respectively, Figure 4b) as compared to the effect of forskolin (see Figure 4a). Thus, after 5 min when the xanthine-stimulated iodide efflux rate was maximal, little change in the cellular cyclic AMP was observed. These results argue against a role for cyclic AMP in mediating the xanthine activation of CFTR.

Effect of forskolin and xanthines on intracellular ATP content

Since CFTR channel activity is dependent on the interaction of ATP with the nucleotide binding folds (NBF, for review see Hanrahan *et al*., 1995), we compared the effect of forskolin and

various substituted xanthines on the ATP content of CFTR(+) CHO cells. In resting cells, the level of ATP was 51 ± 5 nmol mg^{-1} protein ($n=6$). The effect of forskolin or xanthine derivatives is expressed as % of ATP content of cells incubated in the absence of drugs. Forskolin (5 μM, $96 \pm 8\%$, $n=4$) or the following xanthines (500 μM) IBMX (X-17, $102 \pm 6\%$, $n=4$), theophylline (X-06, $118 \pm 5\%$, $n=2$), caffeine (X-11, $108 \pm 1\%$, $n=2$), enprofylline (X-14, $128 \pm 5\%$, $n=2$), DPX (X-20, $100 \pm 11\%$, $n=2$), 8-phenyltheophylline (X-19, $117 \pm 19\%$, $n=4$), DPMX (X-25, $113 \pm 19\%$, $n=4$) have no

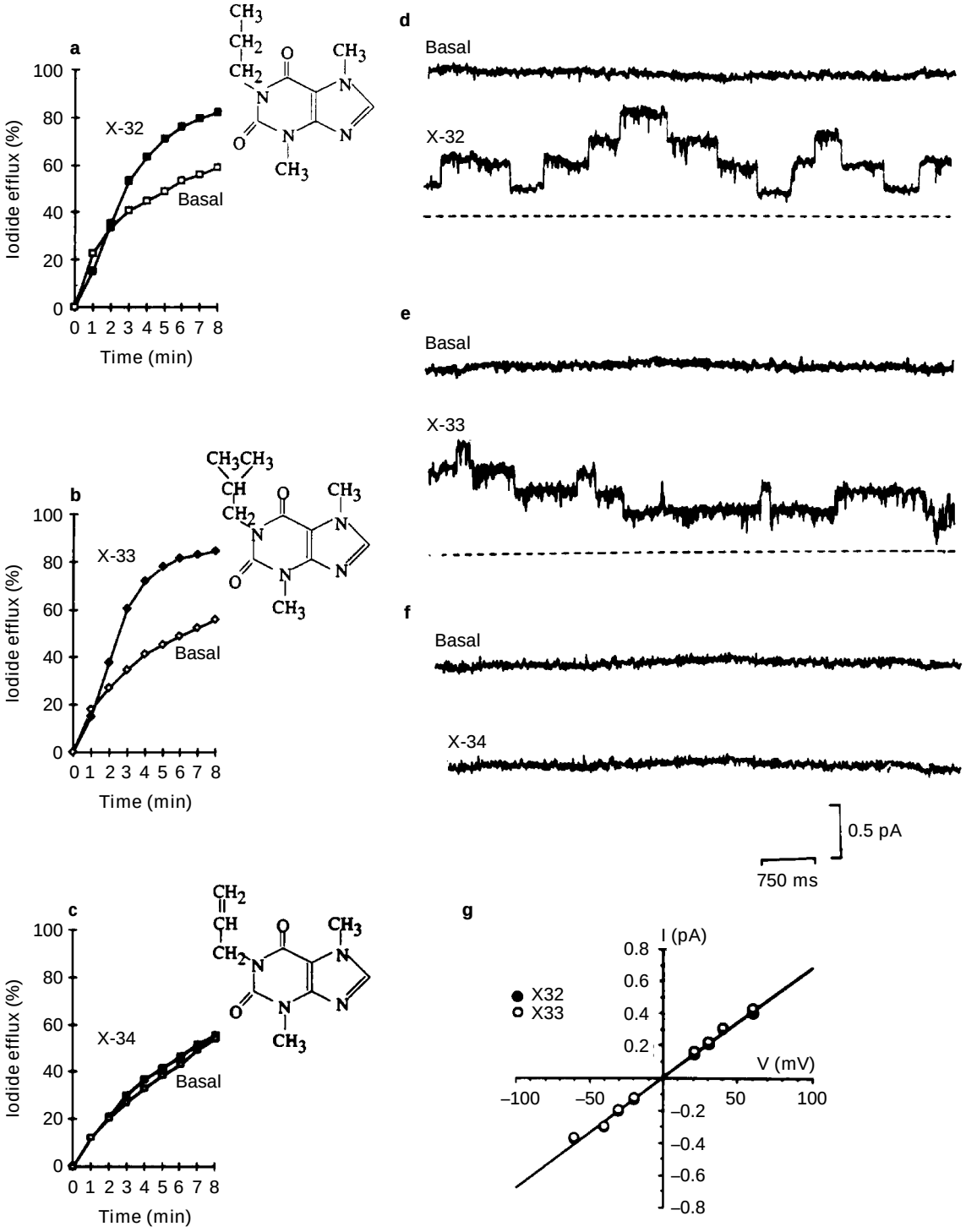

Figure 3 Synthesized xanthines stimulated iodide efflux and opened CFTR channels. (a), (b), and (c) Chemical structure of the xanthines tested and ^{125}I efflux as a function of time from CFTR(+) CHO cells in the presence or absence (basal) of 500 μM xanthines as indicated. (a) 3,7-dimethyl-1-propylxanthine (X-32); (b) 3,7-dimethyl-1-isobutylxanthine (X-33); (c) 3,7-dimethyl-1-(2-propenyl) xanthine (X-34); note that X-34 failed to stimulate the iodide efflux. (d) and (e) Representative recordings of CFTR channels in the cell-attached configuration ($-V_{pipette} = -30$ mV) activated by 500 μM xanthine as indicated (bottom trace); note the absence of channel activity before drug addition (noted basal, upper trace for each panel). (f) The xanthine derivative X-34 (500 μM) failed to activate CFTR in the cell-attached configuration ($-V_{pipette} = -30$ mV). (g) Corresponding current-voltage relationship for channels activated by X-32 and X-33 as indicated. V represents $-V_{pipette}$ in mV.

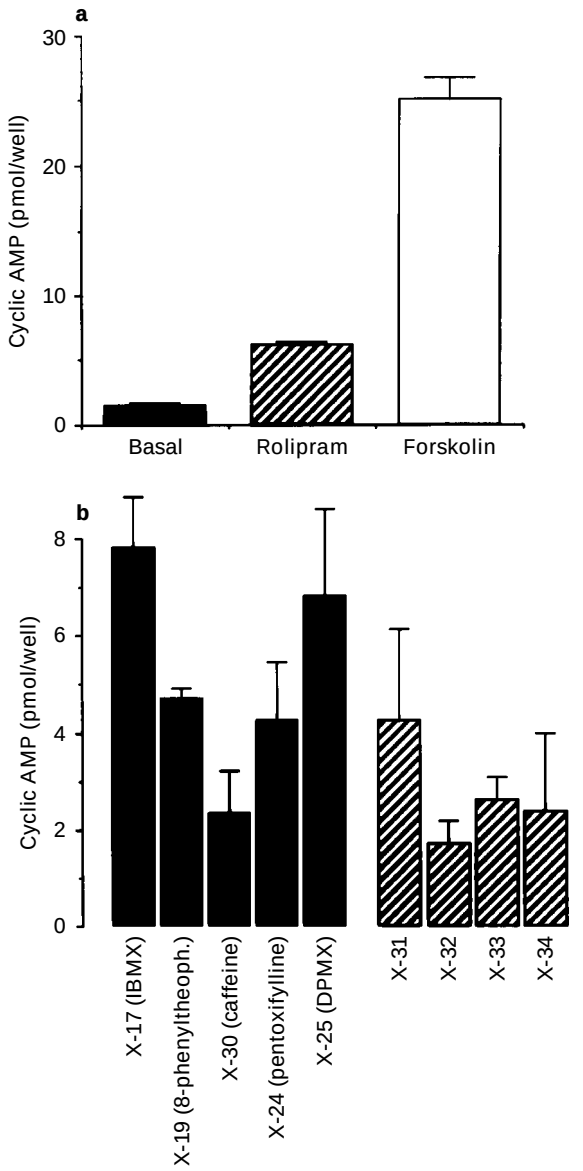

Figure 4 Intracellular cyclic AMP in CFTR(+) CHO cells. (a) Intracellular cyclic AMP content in resting cells (basal) and during stimulation with forskolin (5 μM) or rolipram (100 μM). (b) Summary of cyclic AMP contents in the presence of various xanthine derivatives as indicated (all xanthines at 500 μM). Data are expressed as pmol cyclic AMP/well and are presented as mean$\pm$s.d. for 3 separate determinations. Basal cellular cyclic AMP content was 1.4$\pm$0.3 pmol cyclic AMP/well.

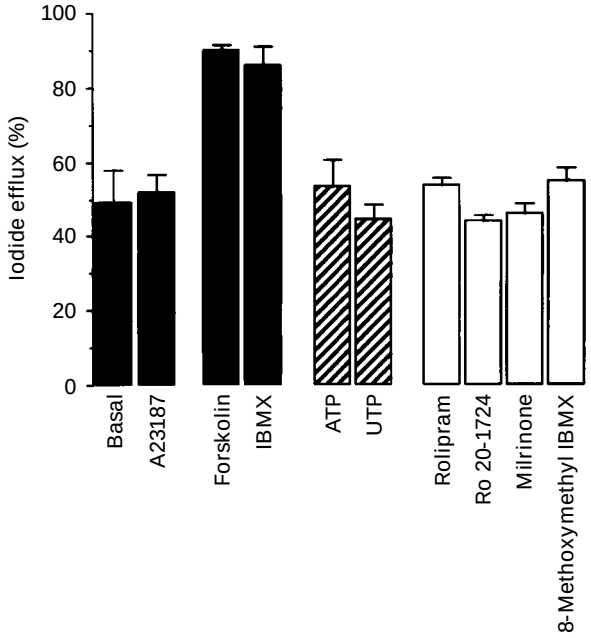

Figure 5 Lack of stimulation of iodide efflux by nucleotides and phosphodiesterase inhibitors. Summary of cumulative iodide efflux after 5 min (T5) from CFTR(+) CHO cells in the presence of the calcium ionophore A23187 (10 μM), forskolin (5 μM), IBMX (500 μM), ATP or UTP (both at 100 μM) and the cyclic AMP-dependent phosphodiesterase (PDE) inhibitors rolipram (100 μM) or Ro 20-1724 (100 μM), the cyclic GMP-dependent PDE inhibitor milrinone (100 μM) or the calcium-calmodulin-dependent PDE inhibitor 8-methoxymethyl IBMX (100 μM).

effect on the ATP content of CHO cells. Although we only determined the total cellular ATP content of CFTR(+) CHO cells and did not take into consideration possible subcellular pools, these data suggest that forskolin and xanthines do not stimulate CFTR channels through modulation of cellular ATP. In addition, extracellular ATP (100 μM) or UTP (100 μM) did not activate the iodide efflux from CFTR(+) CHO cells (Figure 5), or stimulate CFTR channels in the cell-attached configuration (not shown).

Discussion

In the present study we used a rapid assay to screen a series of modified xanthines and found a correlation between their potency as CFTR activators and the position and nature of the substituents in the xanthine skeleton. Xanthines are interesting pharmacological tools because they are amenable to detailed structure-activity studies (Croce *et al*., 1979; Bruns *et al*., 1980; 1983; Snyder *et al*., 1981; Sakai *et al*., 1992; Miyamoto *et al*., 1993) and because they are potent activators of CFTR channel activity in a variety of cell types (Becq *et al*., 1993a; 1994) as well as in *Xenopus* oocytes injected with cRNA-CFTR (Drumm *et al*., 1991).

We have identified DPMX, pentoxifylline as novel and potent activators of CFTR chloride channels. DPMX also stimulates CFTR channel activity in reverted CFPAC cells (D. Escande, I. Baro and F. Becq, unpublished data), in the colonic cell line HT29, and in CFTR-expressing oocytes (F. Becq and B. Verrier, unpublished data). Interestingly, *in vivo* administration of theophylline, aminophylline or pentoxifylline (three activators of CFTR) has proven to be of benefit (as bronchodilators) in the treatment of pulmonary infection of CF patients (Larsen *et al*., 1980; Isles *et al*., 1983; Knoppert *et al*., 1988; Pan *et al*., 1989). Moreover, pentoxifylline may have efficacy as a therapeutic agent for CF lung disease because of its effects on neutrophils (Aronoff *et al*., 1994). Our results showing that these agents also activate the CFTR channel is intriguing and requires further investigation as to the role of CFTR in pulmonary infection.

Substituted xanthines have been used previously as inhibitors of phosphodiesterases (PDE) (Ukena *et al*., 1993; Miyamoto *et al*., 1995), and alkaline phosphatases (Croce *et al*., 1979), and as adenosine-receptors antagonists (Bruns *et al*., 1980; 1983; Snyder *et al*., 1981; Ukena *et al*., 1993). The structure-activity relationship depends on whether a xanthine is used as a CFTR activator, PDE inhibitor or adenosine receptor antagonist. The novel structure-activity described in

this study for xanthine compounds as activators of CFTR may be useful for the design and synthesis of potent CFTR chloride channel openers.

Lack of correlation between phosphodiesterase inhibition and CFTR opening

The following findings show that activation of CFTR by xanthines is not correlated with phosphodiesterase inhibition: (i) the specific PDE inhibitors rolipram, Ro 20-1724 and milrinone failed to open CFTR channels, (ii) among the non-specific PDE inhibitors IBMX, theophylline, caffeine, 8-cyclopentyl theophylline, 1,3-diethyl-8-phenyl-xanthine and DPMX ($EC_{50} = 10-600$ μM, Evoniuk *et al*., 1987; Ukena *et al*., 1993) only IBMX, theophylline and DPMX were able to open CFTR, and (iii) the ability of xanthines to increase cyclic AMP concentration was not correlated with their ability to open CFTR. For example, rolipram and DPMX increased the cyclic AMP concentration to a similar level, but only DPMX opened CFTR. These results strongly suggest that xanthine-induced activation of CFTR does not involve inhibition of PDE. A similar conclusion was drawn by Simasko & Yan (1993), who showed that IBMX activates voltage-dependent calcium currents by a cyclic AMP-independent mechanism in endocrine cells. Similarly, the effect of IBMX or theophylline on the voltage-activated chloride current of toad skin cannot be explained solely by inhibition of PDE (Katz & Nagel, 1995).

Our data show that rolipram clearly elevates cyclic AMP in CFTR(+) CHO cells; since it is known that CFTR is activated by increases in cyclic AMP and PKA-dependent phosphorylation (see Hanrahan *et al*., 1995), why is there no detectable increase in CFTR activity? One would predict a cyclic AMP effect and an additional direct xanthine effect. Since many investigators have used mixtures containing IBMX to increase cyclic AMP levels, might such manipulations be primarily due to a direct xanthine-stimulation of CFTR? Since it has also been proposed that IBMX, theophylline and more specific phosphatase inhibitors activate CFTR through the inhibition of membrane associated phosphatases (Tabcharani *et al*., 1991; Becq *et al*., 1994; 1996b), more likely explanations for the effect of X-32, X-33 and the other xanthine derivatives identified as activators of CFTR would be either a direct effect on CFTR or an indirect action via some channel-associated phosphatases. In both cases, a pure cyclic AMP effect does not explain by itself the activation of CFTR by these drugs. Indeed, Logan *et al*. (1993) showed that IBMX can bind on the first nucleotide binding domain of CFTR. Direct interaction of IBMX with a potassium channel has also been proposed in a study on rat sensory neurones (Usachev *et al*., 1995).

Lack of correlation between adenosine-receptor antagonist and CFTR opening

Substitution at C8 of xanthine is important to obtain highly potent and specific adenosine receptors antagonists (Bruns *et al*., 1980; 1983; Snyder *et al*., 1981). On the contrary, we found that this position must be kept free for CFTR activation. Indeed the xanthines X-19, X-20, X-22 and X-23 are potent adenosine receptors antagonists but all failed to stimulate CFTR. Thus by manipulating the 8-position of xanthine, we can readily discriminate between the two functions, i.e. activation of CFTR or A-receptor antagonism. Moreover, the CHO-K1 cell used in this study does not express endogenous A receptors (Hill *et al*., 1997). In agreement with our conclusion, Jacobson *et al*. (1995) recently concluded from a structure-activity study that the activation of mutant CFTR by CPX (X-23 in this study) does not involve adenosine receptors.

Varying alkyl substitutions in the xanthine skeleton correlate with CFTR activation

Based on the present results, two families of xanthine compounds can be defined. The first family comprised 1,3-dialkyl xanthine derivatives (theophylline, IBMX and aminophyline). Small changes in the chemical structure of the activator theophylline produced significant pharmacological changes; e.g. demethylation produced the inactive 3-methyl xanthine. Additional substitution at N7 of theophylline yielded caffeine which is inactive on CFTR. Any substitution at C8 abolished the effect of the xanthine on CFTR, although 8-substituted xanthines are highly potent adenosine receptor antagonists (Bruns *et al*., 1980; 1983; Snyder *et al*., 1981; Jacobson *et al*., 1993). The second family is composed of 1,3,7-trialkyl xanthines (DPMX, pentoxifylline, X-32 and X-33). Slight chemical modification of the alkyl group at N1 abolished the potency of the compound as activator of CFTR. For example, switching a propyl group at N1 (X-32) to a propenyl group (X-34) prevented activation of CFTR.

Design and synthesis of novel openers of CFTR chloride channels

The goal of our study was to examine possible strategies for constructing an opener of the CFTR channel. For example, based on a structure activity relationship study, the development of safer xanthine drugs has been explored for the treatment of obstructive airway disease (Persson, 1986). Here, we present a structure-activity relationship study on activators of CFTR, and describe the successful activation of CFTR by a series of novel synthesized trialkyl xanthines (X-32 and X-33) which act without altering the cellular cyclic AMP level. Moreover, manipulating the nature and position of the substituents yielded both the active and inactive forms of the derivative (X-31 is the inactive form of X-32). Based on this analysis, a summary of xanthine derivatives of CFTR activators is presented in Figure 6. Non-substituted C8 and N9 positions are required. Substitutions at positions N1, N3 and N7 are crucial for CFTR activations. This scheme should help the design of more potent CFTR activators which may

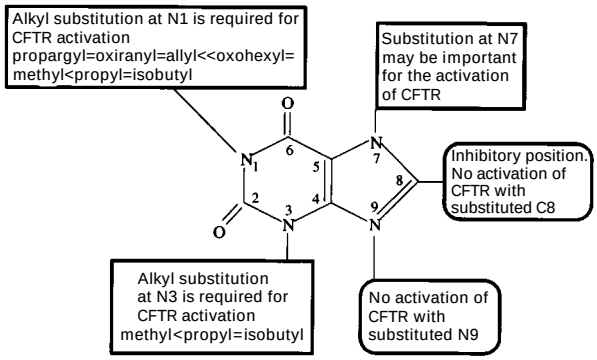

Figure 6 Summary of the structural basis for specificity and potency of xanthine derivatives as activators of CFTR. Substitution at both N1 and N3 are essential for CFTR channel activation. The nature of the chemical group located at the N1 or N3 is also an important determinant of potency. Substituting N7 may be important in activating CFTR, except when methyl groups are located at N1 and N3 (see text for details). Occupancy of the C8 or N9 generates inactive derivatives.

provide convenient tools for studying chloride channels in native tissues.

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References

ARONOFF, S.C., QUINN, F.J., CARPENTER, L.S. & NOVICK, W.J. (1994). Effects of pentoxifylline on sputum neutrophil elastase and pulmonary function in patients with cystic fibrosis; preliminary observations. *J. Pediatr.,* **125,** 992 – 997.

BECQ, F., FANJUL, M., MERTEN, M.D., FIGARELLA, C., HOLLANDE, E. & GOLA, M. (1993a). Possible regulation of CFTR-chloride channels by membrane-bound phosphatases in pancreatic duct cells. *FEBS. Letts.,* **327,** 338 – 342.

BECQ, F., JENSEN, T.J., CHANG, X.-B., SAVOIA, A., ROMMENS, J.M., TSUI, L.-C., BUCHWALD, M., RIORDAN, J.R. & HANRAHAN, J.W. (1994). Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 9160 – 9164.

BECQ, F., MERTEN, M.D., VOELCKEL, M.A., GOLA, M. & FIGARELLA, C. (1993b). Characterization of cAMP dependent CFTR-chloride channels in human tracheal gland cells *FEBS Lett.,* **321,** 73 – 78.

BECQ, F., METTEY, Y., VIERFOND, J.M., CHAPPE, V., PIN, T., HANRAHAN, J.R., RIORDAN, J.R., GOLA, M. & VERRIER, B. (1996a). Designing and synthesis of openers of the cystic fibrosis chloride channel. *Ped. Pulmonol.,* **13,** 284.

BECQ, F., VERRIER, B., CHANG, X.-B., RIORDAN, J.R. & HANRAHAN, J.W. (1996b). cAMP- and Ca^{2+}-independent activation of cystic fibrosis transmembrane conductance regulator channels by phenylimidazothiazole drugs. *J. Biol. Chem.,* **271,** 16171 – 16179.

BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1980). Adenosine receptors in brain membranes: binding of N^6-cyclohexyl [^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]phenylxanthine. *Proc. Natl. Acad. Sci. U.S.A.,* **77,** 5547 – 5551.

BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1983). Adenosine receptor binding: structure-activity analysis generates extremely potent xanthine antagonists. *Proc. Natl. Acad. Sci. U.S.A.,* **80,** 2077 – 2080.

CHANG, X.-B., TABCHARANI, J.A., HOU, Y.-X., JENSEN, T.J., KARTNER, N., ALON, N., HANRAHAN, J.W. & RIORDAN, J.R. (1993). Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all ten PKA consensus phosphorylation sites. *J. Biol. Chem.,* **268,** 11304 – 11311.

COTTAM, H.B., SHIH, H., TEHRANI, L.R., WASSON, D.B. & CARSON, D.A. (1996). Substituted xanthines, pterinediones, and related compounds as potential antiinflammtory agents. Synthesis and biological evaluation of inhibitors of tumor necrosis factor α. *J. Med. Chem.,* **39,** 2 – 9.

CROCE, M.A., KRAMER, G.L. & GARBERS, D.L. (1979). Inhibition of alkaline phosphatase by substituted xanthines. *Biochem. Pharmacol.,* **28,** 1227 – 1231.

DALEMANS, W., BARBRY, P., CHAMPIGNY, G., JALLAT, S., DOTT, K., DREYER, D., CRYSTAL, R.G., PAVIRANI, A., LECOCQ, J.P. & LAZDUNSKI, M. (1991). Altered chloride ion channel kinetics associated with delta F508 cystic fibrosis mutation. *Nature,* **354,** 526 – 528.

DALY, J.W., PADGETT, W.L. & SHAMIM, M.T. (1986). Analogues of caffeine and theophylline: effect of structural alterations on affnity at adenosine receptors. *J. Med. Chem.,* **29,** 1305 – 1308.

DRUMM, M.L., WILKINSON, D.J., SMIT, L.S., WORRELL, R.T., STRONG, T.V., FRIZZELL, R.A., DAWSON, D.C., & COLLINS, F.S. (1991). Chloride conductance expressed by delta F508 and other mutant CFTRs in *Xenopus* oocytes. *Science,* **254,** 1797 – 1799.

EIDELMAN, O., GUAY-BRODER, C., VAN GALLEN, P.J.M., JACOBSON, K.A., FOX, C., TURNER, R.R.J., CABANTCHIK, Z.I. & POLLARD, H.B. (1992). A1 adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 5562 – 5566.

EVONIUK, G., JACOBSON, K.A., SHAMIM, M.T., DALY, J.W. & WURTMAN, R.J. (1987). A1- and A2-selective adenosine antagonists: *In vivo* characterization of cardiovascular effects. *J. Pharmacol. Exp. Ther.,* **242,** 882 – 887.

HANRAHAN, J.W., TABCHARANI, J.A., BECQ, F., MATHEWS, C.J., AUGUSTINAS, O., JENSEN, T.J., CHANG, X.-B. & RIORDAN, J.R. (1995). Function and dysfunction of the CFTR chloride channel. In *Ion Channels and Genetic Diseases*, Society of General Physiologists Symposium Series, Vol.50, ed. Dawson, D.C. & Frizzell, R.A. pp. 125 – 137, Rockfeller Univ. Press.

HILL, R.J., OLEYNEK, J.J., HOTH, C.F., RAVI KIRON, M.A., WENG, W., WESTER, R.T., TRACEY, W.R., KNIGHT, D.R., BUCHHOLZ, R.A. & KENNEDY, S.P. (1997). Cloning, expression and pharmacological characterization of rabbit adenosine A1 and A3 receptors. *J. Pharmacol. Exp. Ther.,* **280,** 122 – 128.

ISLES, A., SPINO, M., TABACHNIK, E., LEVISON, H., THIESSEN, J. & MACLEOD, S. (1983). Theophylline disposition in cystic fibrosis. *Am. Rev. Respir. Dis.,* **127,** 417 – 421.

JACOBSON, K.A., GALLO-RODRIGUEZ, X., MELMAN, N., MAILLARD, M., VAN, BERGEN, A., VAN GALLEN, P.J.M. & KARTON, Y. (1993). Structure-activity relationships of 8-styrylxanthines as A2-selective adenosine antagonists. *J. Med. Chem.,* **36,** 1333 – 1342.

JACOBSON, K.A., GUAY-BRODER, C., VAN GALLEN, P.J.M., GALLO-RODRIGUEZ, C., MELMAN, N., MAILLARD, M., JACOBSON, M.A., EIDELMAN, O. & POLLARD, H.B. (1995). Stimulation by alkylxanthine of chloride efflux in CFPAC-1 cells does not involve A1 adenosine receptors. *Biochemistry,* **34,** 9088 – 9094.

KATZ, U. & NAGEL, W. (1995). Effect of cyclic AMP and theophylline on chloride conductance across toad skin. *J. Physiol.,* **489,** 105 – 114.

KNOPPERT, D.C., SPINO, M., BECK, R., THIESSEN, J.J. & MACLEOD, S. (1988). Cystic fibrosis: enhanced theophylline metabolism may be linked to the disease. *Clin. Pharmacol. Ther.,* **44,** 254 – 264.

KOCH, M.C., STEINMEYER, K., LORENZ, C., RICKER, K., WOLF, F., OTTO, M., ZOLL, B., LEHMANN-HORN, F., GRZESCHIK, K.H. & JENTSCH, T.J. (1992). The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science,* **257,** 797 – 800.

LARSEN, G.L., BARRON, R.J., LANDAY, R.A. & COTTON, E.K. (1980). Intravenous aminophylline in patients with cystic fibrosis. Pharmacokinetics and effects on pulmonary function. *Am. J. Dis. Child.,* **134,** 1143 – 1148.

LOGAN, J., HIESTAND, D., HUANG, Z., MUCCIO, D., HALEY, B. & SORSCHER, E.J. (1993). 3-Isobutyl-1-methylxanthine (IBMX) blocks binding of ATP analogs to the CFTR first nucleotide binding domain (NBD-1). *FASEB J.,* **7,** A426.

MIYAMOTO, K.-I., SAKAI, R., KURITA, M., OHMAE, S., SANAE, F., SAWANISHI, H., HASEGAWA, T. & TAKAGI, K. (1995). Effects of alkyl substituents of xanthine on phosphodiesterase isoenzymes. *Biol. Pharm. Bull.,* **18,** 431 – 434.

MIYAMOTO, K.-I., YAMAMOTO, Y., KURITA, M., SAKAI, R., KONNO, K., SANAE, F., OHSHIMA, T., TAKAGI, K., HASEGAWA, T., IWASAKI, N., KAKIUCHI, M. & KATO, H. (1993). Bronchodilator activity of xanthine derivatives substituted with functional groups at the 1- or 7-position. *J. Med. Chem.,* **36,** 1380 – 1386.

PAN, S.-H., CANAFAX, D.M., LE, C.T., CIPOLLE, R.J., UDEN, D.L. & WARWICK, W. (1989). Bronchodilation from intravenous theophylline in patients with cystic fibrosis: results of a blinded placebo-controlled crossover clinical trial. *Pediatr. Pulmonol.,* **6,** 172 – 179.

PERSSON, C.G.A. (1986). Development of safer xanthine drugs for treatment of obstructive airway disease. *J. Allergy Clin. Immunol.,* **78,** 817 – 824.

RIORDAN, J.R., ROMMENS, J.M., KEREM, B.-S., ALON, N., ROZMAHEL, R., GRZELCZAK, Z., ZIELENSKI, J., LOK, S., PLAVSIC, N, CHOU, J.-L., DRUMM, M.L., IANNUZZI, M.C., COLLINS, F.S. & TSUI, L.-C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science,* **245,** 1066 – 1072.

SAKAI, R., KONNO, K., YAMAMOTO, Y., SANAE, F., TAKAGI, K., HASEGAWA, T., IWASAKI, N., KAKIUCHI, M., KATO, H. & MIYAMOTO, K.-I. (1992). Effects of alkyl substitutions of xanthine skeleton on bronchodilation. *J. Med. Chem.,* **35,** 4039 – 4044.

SIMASKO, S.M. & YAN, S. (1993). 3-Isobutyl-1-methylxanthine inhibits sustained calcium current independently of cyclic AMP in neuronal and endocrine cells. *Molec. Pharmacol.,* **44,** 622 – 627.

SNYDER, S.H., KATIMS, J.J., ANNAU, Z., BRUNS, R.F. & DALY, J.W. (1981). Adenosine receptors and behaviour actions of methylxanthines. *Proc. Natl. Acad. Sci. U.S.A.,* **78,** 3260 – 3264.

STEINMEYER, K., SCHWAPPACH, B., BENS, M., VANDEWALLE, A. & JENTSCH, T.J. (1995). Cloning and functional expression of rat CLC-5, a chloride channel related to kidney disease. *J. Biol. Chem.,* **270,** 31172 – 31177.

TABCHARANI, J.A., CHANG, X-.B., RIORDAN, J.R. & HANRAHAN, J.W. (1991). Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature,* **352,** 628 – 631.

TSUI, L.-C. & BUCHWALD, M. (1991). Biochemical and molecular genetics of cystic fibrosis. *Adv. Human Genetic.,* **20,** 153 – 266.

UKENA, D., SCHUDT, C. & SYBRECHT, G. (1993). Adenosine receptor blocking xanthines as inhibitors of phosphodiesterase isozymes. *Biochem. Pharmacol.,* **45,** 847 – 851.

USACHEV, Y., KOSTUYK, P. & VERKHRATSKY, A. (1995). 3-isobutyl-1-methylxanthine (IBMX) affects potassium permeability in rat sensory neurones via pathways that are sensitive and insensitive to $[Ca^{2+}]_{in}$. *Pflügers Arch.-Eur. J. Physiol.,* **430,** 420 – 428.

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