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THEOPHYLLINE-LIKE PROPERTIES OF XANTHINE ANALOGS

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Abstract—Theophylline and other methylxanthines display a large number of biological effects, some of which are clinically important. The effects of these compounds are commonly ascribed to an inhibition of cyclic AMP breakdown. However, it becomes actually evident that another mechanism, namely adenosine receptor antagonism, could be responsible for certain methylxanthine effects. It could be of interest to find new compounds displaying only one of these mechanisms, either phosphodiesterase inhibition or adenosine receptor antagonism. We have studied several synthetic imidazo[1,2a]pyrazines, some of which display theophylline-like pharmacological properties at lower doses than theophylline. We showed that some of these compounds inhibited mitogen-induced [^{3}H]-thymidine uptake by human lymphocytes, which is consistent with increases in cyclic AMP levels: the most efficient compounds were those which were better phosphodiesterase inhibitors than theophylline and poorer adenosine receptor antagonists.

Theophylline and other methyl-xanthines display a large number of biological effects, some of which are clinically important [1]. Theophylline is one of the mainstays of therapy in asthma. However, its therapeutic use must take into account its relatively high toxicity and its numerous side effects, which necessitate individual pharmacokinetic studies and seric determinations when high or repeated doses must be used.

The effects of these compounds are commonly ascribed to an inhibition of cyclic AMP breakdown. The ubiquitousness of cyclic AMP and its phosphodiesterases could explain why the methyl-xanthines affect so many different tissues and cells. Inhibition of cyclic AMP phosphodiesterase could also explain why methyl-xanthines are capable of potentiating the action of several hormones. However, it becomes actually evident that another mechanism, namely antagonism of adenosine receptors, could be responsible for certain methylxanthine effects [1–5]. It could be of interest to find new compounds displaying only one kind of theophylline property, either phosphodiesterase inhibition or adenosine antagonism. Several attempts have been made in this direction [6–8].

We have studied several imidazo[1,2a]pyrazines, the synthesis and pharmacological properties of which have been reported elsewhere [19]. Some of these xanthine analogs were found to be spasmolytic and bronchodilatator at lower doses than theophylline.

Theophylline was recently found to exert a specific action on lymphocytes and could be used to characterize and isolate T lymphocytic subpopulations with different functions [10–15]: theophylline-resistant (T_R) lymphocytes which act as inducers/helpers of B lymphocyte differentiation and theophylline-sensitive (T_S) lymphocytes acting as suppressor of B lymphocyte differentiation.

We compared the activity of our xanthine analogs to that of theophylline on human lymphocytes. We have previously shown the occurrence of adenosine receptors coupled to adenylate cyclase on lymphocyte plasma membranes [16–17]; these receptors are of the R_a type [18], leading to adenylate cyclase stimulation, and are anatogonized by theophylline. Theophylline is also a phosphodiesterase inhibitor in lymphocytes [19] like in other cell types.

We reported here the effects of theophylline and imidazo[1,2a]pyrazines on cyclic AMP phosphodiesterases, on 5′*N*-ethylcarboxamide adenosine (NECA)-stimulated adenylate cyclase, and on [^{3}H]-thymidine incorporation in lymphocytes stimulated by various mitogens.

MATERIALS AND METHODS

Cells. Lymphocytes were obtained from 20- to 40-year-old healthy donors (men and women). Heparinized blood was centrifuged on MSL (lymphocyte separation medium) from Eurobio. The lymphocytes were resuspended in RPMI 1640 medium supplemented with 15% fetal calf serum and antibiotics.

Mouse thymocytes were prepared from 4- to 5-week male Swiss mice. Thymuses were removed, the cells were teased apart in Hanks medium, filtered through nylon screen and washed twice with Hanks medium, then with 10 mM Tris–HCl, pH 7.5, NaCl 0.15 M.

Immature mouse thymocytes were obtained by

selective agglutination with peanut agglutinin PNA, as previously described [20].

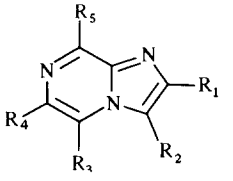
Cell homogenates. Pelleted mouse thymocytes were resuspended in hypotonic solution (10 mM Tris–HCl, pH 7.5) for a few minutes, with two gentle strokes in a Dounce homogenizor. The disrupted cells were filtered through nylon gauze to discard the aggregated material and the filtrate was taken as homogenate for adenylate cyclase and phosphodiesterase determination.

Cell cultures. Human lymphocytes (1.2×10^6 cells per ml) were cultured in microtest II plates (Falcon), 0.2 ml per well, at 37°, under a wet CO_2–air (5:95) atmosphere. The incubation lasted 3 days in the case of phytohemagglutinin (PHA) or concanavalin A (Con A) stimulation, and 5 days in the case of pokeweed mitogen (PWM) stimulation. The blastic transformation was determined by measuring the uptake of [^{3}H]-thymidine which was added (1 μCi per well) 4 and 18 hr before the end of the incubation period for PHA (or Con A) and PWM stimulation respectively. The radioactivity taken up was measured in triplicate with a liquid scintillation spectrometer and expressed in cpm ± standard deviation. The significance of the results was assessed by Student's *t*-test.

Adenylate cyclase activity. Adenylate cyclase activities were measured by Salomon *et al.* [21] in 100 μl 25 mM Tris–HCl, pH 7.5, containing 20 U/ml creatine phosphokinase, 15 mM creatine phosphate, 2.5 mM $MgCl_2$, 0.2 mM cyclic AMP and 0.1 mM [α-^{32}P]-ATP ($1.5–3 \times 10^6$ cpm per assay). The reaction lasted 10 min at 30° and was stopped by adding 150 μl 10 mM Tris–HCl, pH 7.5, containing 2.5 mM ATP, 50 μM [^{3}H]-cyclic AMP ($\sim 10^4$ cpm per assay) and by heating the tubes for 3 min. Under all described conditions cyclic AMP production was proportional to the reaction time and the enzyme amount. The preincubation of cell homogenates with effectors lasted 5 min at 30°.

Phosphodiesterase activity. Phosphodiesterase activities were determined on immature PNA$^+$ mouse thymocytes (which represent about 90% of the whole thymocyte population), using cyclic [^{3}H]-AMP as substrate, in 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, at 30° with 15–20 μg homogenate proteins per assay. The reaction time varied with experiments: in all cases hydrolysed cyclic AMP was proportional to time and protein amount. Resulting 5′[^{3}H]-AMP was separated from excess substrate by thin layer chromatography on silica gel plates (Merck ref. 5567, elution with propanol, methanol, 34% ammonia, water 45/15/20/20) and measured by radioactivity counting. As cyclic AMP and adenosine have identical R_f values under these conditions, control experiments were carried out and showed that further degradation of 5′[^{3}H]-AMP into [^{3}H]-adenosine and [^{3}H]-inosine was negligible for PNA$^+$ thymocytes: no difference was observed in the amount of 5′[^{3}H]-AMP in the presence or in the absence of 5′nucleotidase inhibitors. Phosphodiesterase activities were also determined in the presence of excess exogenous adenosine deaminase: no inosine formation (the R_f of which is largely different from those of other nucleosides and nucleotides) could be evidenced, which shows that adenosine formation during the assay is negligible. This is consistent with the low 5′nucleotidase activity of mouse PNA$^+$ thymocytes [22].

Materials. The synthesis and chemical characterization of the following imidazo[1,2a]pyrazines were reported elsewhere [9]:



	R_1	R_2	R_3	R_4	R_5
SC-0	H	H	H	H	H
SC-8	CN	H	H	Br	Br
SC-9	CH_3	CO_2Et	H	H	H
SC-12	CH_2OH	H	H	H	H
SC-15	H	H	H	Br	Br
SC-16	H	H	Br	H	H
SC-17	CO_2Et	H	Br	H	H
SC-21	NH_2	Br	H	H	H
SC-50	Cl	$COCH_3$	H	H	H
SC-51	CH_2CO_2Et	H	H	Br	Br

SCHEME 1

Creatine phosphate, creatine phosphokinase were obtained from Boehringer, nucleotides and chloro-2-adenosine from Sigma, [α-^{32}P]-ATP from Amersham Radiochemical Centre, [^{3}H]-cyclic AMP, [^{3}H]-thymidine from CEN Saclay. 5′*N*-ethylcarboxamide adenosine was kindly supplied by Dr Schick (Byk Gulden Pharmazeutika, Constanz, Germany).

RESULTS AND DISCUSSION

Phosphodiesterase activities were determined on mouse PNA$^+$ thymocytes using an easy and fast method. With this type of cell, which almost completely lacks 5′nucleotidase activity [22], it is possible

Table 1. Inhibition of cyclic AMP phosphodiesterase in mouse thymocytes

Inhibitor (200 μM)	Inhibition (%)
None	0
Theophylline	35
SC-51	76
SC-9	73
SC-8	67
SC-16	19
SC-15	18
SC-21	16
SC-50	10
SC-12	7
SC-0	0

Phosphodiesterase was determined as in Materials and Methods with 1.14 μM cyclic AMP and 15–20 μg proteins per assay. The reaction lasted 16 min. The specific activity in the absence of inhibitor was 4 nM/mg/min.

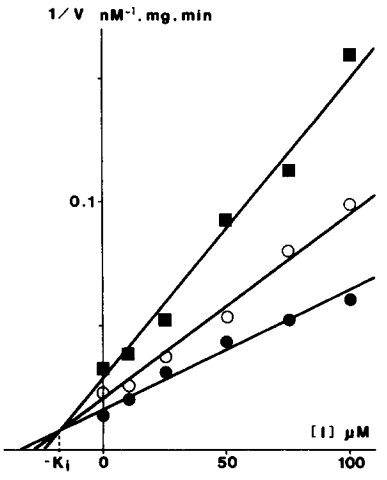


Fig. 1. Dixon plots of cyclic AMP phosphodiesterase inhibition by SC-51. Cyclic AMP phosphodiesterase activities in mouse thymocytes as in Materials and Methods with three substrate concentrations (0.425 μM (■——■), 0.85 μM (○——○) and 1.7 μM (●——●)) in the presence or in the absence of various inhibitor SC-51 (I) concentrations.

to separate 5′[^{3}H]-AMP resulting from cyclic [^{3}H]-AMP splitting by the enzyme without further hydrolysis to [^{3}H]-adenosine. A simple thin layer chromatography allows the separation of 5′[^{3}H]-AMP from excess substrate, while it is difficult to separate [^{3}H]-adenosine from cyclic [^{3}H]-AMP under these conditions. We controlled that, in the presence of 5′nucleotidase inhibitors (such as concanavalin A), the amount of recovered 5′[^{3}H]-AMP was unchanged. We checked also that no [^{3}H]-inosine resulting from [^{3}H]-adenosine deamination was produced under the assay conditions. The specific activity of cyclic AMP phosphodiesterase in mouse thymocytes was about 4 nM/mg/min; K_m for cyclic AMP high affinity sites was found to be 0.83 μM.

Table 1 presents phosphodiesterase inhibition by 200 μM theophylline or several imidazo[1,2a]pyrazines. The unsubstituted compound (SC-O) has no inhibitory properties. Five compounds (SC-12, SC-15, SC-16. SC-21, SC-50) are clearly less inhibitory than theophylline, while three compounds have twice the inhibitory strength of theophylline (SC-8, SC-9, SC-51). The inhibition constant (K_i) was determined for SC-51, using Dixon's method (Fig. 1); it was about 18 μM.

Table 2. Inhibition of NECA-stimulated adenylate cyclase in mouse thymocytes

Inhibitor (200 μM)	Adenylate cyclase activity (%)
None	100
Theophylline	17
SC-9	47
SC-21	48
SC-51	49
SC-8	57
SC-0	77
SC-50	78
SC-15	90
SC-16	90
SC-12	97

Adenylate cyclase was determined on cell homogenates (25 μg proteins per assay), as in Materials and Methods, in the presence of 1 μM NECA. The specific activity in the absence of inhibitor was 50 and 150 pmol cyclic AMP/mg/10 min, in the absence or in the presence of 1 μM NECA, respectively.

Adenylate cyclase activities of mouse thymocyte homogenates were determined under conditions where phosphodiesterases were saturated with excess cyclic AMP. It has proved to be the appropriate method to study adenosine receptors coupled to adenylate cyclase since the use of phosphodiesterase inhibitors, such as RO201724 or papaverine, which do not completely block the enzyme, can lead to wrong interpretations [19]. We have previously demonstrated the presence of adenosine receptors of Ra type on mouse thymocytes [16, 17] and shown that NECA was the best agonist: the half-maximal stimulation of adenylate cyclase was obtained with less than 5×10^{-8} M NECA through high affinity receptor sites. Theophylline is an antagonist of these adenosine receptors and 200 μM theophylline induces a 87% inhibition of 1 μM NECA-induced adenylate cyclase activity. We have previously shown that the involvement of endogenous adenosine in NECA stimulation experiments can be ruled out since it is swept out by high adenosine deaminase activities of thymocytes [17]. All imidazo[1,2a]pyrazines tested were found to be poorer antagonists than theophylline (Table 2): SC-12 has no inhibitory effect; SC-0, SC-15, SC-16, SC-50 have effects at least 4-fold lower than theophylline; four compounds (SC-8, SC-9, SC-51, SC-21) are about two times less inhibitory. It should be noticed that three compounds (SC-8, SC-9, SC-51) are two times better inhibitors of cyclic AMP phosphodiesterase and two times poorer adenosine receptor antagonist than theophylline, when tested at 200 μM concentrations, which was one of our early goal in testing this set of compounds. These three compounds display bronchodilatator properties at lower doses than theophylline [9].

As cyclic AMP is well known to inhibit the proliferation of lymphocytes of any origin, we studied the effects of theophylline (200–500 μM), chloro-2-adenosine (5–15 μM) and the xanthine analogs SC-8 and SC-51 (2.5–15 μM) on human peripheral blood lymphocytes (PBL) stimulated by three mitogenic lectins: PHA, Con A, PWM. Theophylline induced a dose-dependent inhibition of Con A- or PHA-induced [^{3}H]-thymidine incorporation (Table 3). Cl-Ado led to similar effect at much lower doses. Theophylline and Cl-Ado effects appeared almost additive. The inhibition of PWM-induced [^{3}H]-thymidine incorporation required higher theophylline concentrations (500 μM); however, lower theophylline concentrations enhanced the inhibition induced by Cl-Ado alone (10–15 μM).

Table 3 presents also the effects of SC-8 on [^{3}H]-thymidine incorporation by human PBL. SC-8 (2.5–15 μM) induced effects similar to those of 200–500 μM theophylline on Con A and PHA-induced stimulation. SC-8 alone inhibited PWM-induced thy-

Table 3. Effects of theophylline, chloro-2-adenosine (C1-Ado), SC-8 and SC-51 on [^{3}H]-thymidine uptake by mitogen-stimulated human PBL

Effector (μM)		Concanavalin A					PHA					PWM				
	Mitogene (μg/ml)	0	10	10	10	10	0	100	100	100	100	0	60	60	60	60
	Cl-Ado (μM)	0	0	5	10	15	0	0	5	10	15	0	0	5	10	15
Theophylline																
0		5	100[1]	73	21	8.5	1.7	100[2]	58	29	15	4.1	100[3]	103	71	42
200			84	43	4.3	0.4	1.2	63	39	11	0.7	24	114	80	48	20
300		1.4	71	42	3.3	0.3		57	33	5	0.8		154	93	27	7.4
400			64	23	1.6	0.1	0.8	36	20	4.4	0.2	3.2	120	72	29	5.5
500		0.6	47	15	1.3	0.1		39	13	2.2	0.3		76	54	25	2
SC-8																
0		11	100[4]	80	60	27	7	100[5]	93	48	12	3.7	100[6]	83	67	49
2.5		0.7	83	70	19	4.5	4.4	98	57	21	2.6	2.2	74	47	69	27
5			79	45	14	1.8		90	64	11	1.6		77	61	31	9.3
10		6.2	58	12	4.5	0.3	1.5	38	5.8	2.4	0.4	2.2	20	16	4.1	0.6
15			7.4	1.4	0.8	0.3		32	1.1	1.2	0.3		1.3	0.9	1.3	1.2
SC-51																
0		7.4	100[7]	71	22	9	8.8	100[8]	96	50	15	6.5	100[9]	84	26	17
2.5		2	77	51	15	2.3	2.7	106	94	32	6.5	3	90	70	21	16
5			61	51	19	2.8		98	92	27	4.7		84	23	12	11
10		4.6	63	47	18	2.9		102	80	31	4.3	4	30	15	12	16
15			62	44	11	1.6	6.2	116	78	39	4.4		20	17	13	13

Results are expressed as percent of mitogen-induced thymidine uptake in the absence of any effector. The reference values were: (1) 313,400 ± 6100; (2) 147,000 ± 5400; (3) 66,300 ± 6500; (4) 273,200 ± 12,000; (5) 272,000 ± 8900; (6) 111,200 ± 8900; (7) 283,100 ± 9000; (8) 219,700 ± 9200; (9) 163,800 ± 11,900. Each value is the mean of 5–6 experiments.

midine incorporation. SC-8 and Cl-Ado together induced inhibition higher than each compound alone.

SC-51 was found as efficient as 200–500 μM theophylline in the range 2.5–15 μM (Table 3). With Con A-induced stimulation SC-51 and theophylline gave similar effects. SC-51 alone had no effect on PHA-induced stimulation. In the presence of Cl-Ado an additive inhibition was again observed with SC-51. With PWM-induced stimulation, SC-51 gave similar effects as SC-8.

CONCLUSION

Theophylline is both an inhibitor of cyclic AMP phosphodiesterase and an antagonist of adenosine receptors. In lymphocytes these two actions lead to opposite effects: the first induced increases of cyclic AMP levels, the second blocks adenosine-induced adenylate cyclase stimulation. Tables 1 and 2 show that 200 μM theophylline inhibits 37% phosphodiesterase activity and blocks 87% of adenylate cyclase stimulation. The compounds SC-8 and SC-51 display theophylline-like activities but are better phosphodiesterase inhibitors and poorer adenosine receptor antagonists.

It appears difficult to establish some structure–activity relationships with the small number of compounds tested. However, some preliminary remarks could be drawn from our data. The best phosphodiesterase inhibitors which have a low adenosine receptor antagonism are closely related compounds: dibromo derivatives (R_4, R_5) with an electrophilic substitution (R_1). Unsubstituted (SC-0) or monosubstituted compounds (SC-12, SC-15, SC-16) are almost devoided of both phosphodiesterase inhibitory property and adenosine receptor antagonism.

The effects of theophylline, SC-8 and SC-51 on mitogen-induced [^{3}H]-thymidine incorporation by human PBL appeared consistent with their phosphodiesterase inhibitory properties rather than their adenosine receptor antagonism. C1-Ado alone induces lymphocyte suppression by increasing cyclic AMP levels through adenosine receptors coupled to adenylate cyclase [17]. Theophylline, SC-8 and SC-51 alone induce lymphocyte suppression by increasing cyclic AMP levels through cyclic AMP phosphodiesterase inhibition and perhaps by inducing specific suppressive populations [10–12]. The addition of theophylline, SC-8 or SC-51 to C1-Ado induced further lymphocyte suppression; the effects are not fully additive. The antagonism for adenosine receptors which should lower C1-Ado-induced lymphocyte suppression is largely overcome by cyclic AMP phosphodiesterase inhibition. It is interesting to notice that the SC-51 dose efficient in lymphocyte suppression is consistent with the K_i value for phosphodiesterase inhibition. As phosphodiesterase is an intracellular enzyme, the comparison of the effects of xanthine analogs and those of theophylline should take into account the differences in cellular uptake of these drugs (we have until now no data on this point); however, the effects of these compounds on lymphocyte proliferation appeared correlated with their phosphodiesterase inhibitory property.

Compounds SC-8 and SC-51 proved to be much more efficient than theophylline in suppressing mitogen-induced lymphocyte stimulation; 2.5–15 μM concentrations led to the same effects as 200–500 μM theophylline. Furthermore, these compounds are more selective regarding their mode of action. At 200 μM, they are twice better phosphodiesterase inhibitors and twice poorer adenosine receptor antagonists than theophylline. As these compounds display low toxicity, they appear of potential therapeutic interest. It was recently shown that "non-blocking" xanthines, such as emprofylline, may be potent bronchodilatators but lack many theophylline-like actions, specially behavorial effects, and it was suggested that universal adenosine antagonism is both unnecessary and undesirable with xanthine antiasthmatics [8].

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