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Effects of Xanthine Derivatives on Lipolysis and on Adenosine 3′,5′-Monophosphate Phosphodiesterase Activity

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SUMMARY

The lipolytic potencies of 64 compounds, mostly xanthine derivatives, were determined in epididymal fat cells. Nine of the compounds of widely varying lipolytic potency were examined as inhibitors of cyclic AMP phosphodiesterase activity. Substantial lipolytic effects were seen at concentrations producing less than 20% inhibition of phosphodiesterase activity. The most active compound, 1-methyl-3-isobutylxanthine, was about 15 times more potent than theophylline in both systems. Over a 20-fold range of concentrations, there was close agreement between the lipolytic activities of the compounds and their activities as inhibitors of phosphodiesterase. The close correlation between these two activities strongly indicates that the lipolytic activity of the xanthine derivatives is a result of inhibition of cyclic AMP phosphodiesterase.

INTRODUCTION

The methylxanthines caffeine and theophylline stimulate lipolysis in epididymal fat pads and isolated fat cells of the rat (1–3). Adenosine 3′,5′-monophosphate (cyclic AMP) and a variety of hormones which stimulate the formation of cyclic AMP in fat cells also stimulate lipolysis (4, 5); furthermore, both the lipolytic effects of submaximal doses of these hormones and their effects on cyclic AMP levels in fat cells are potentiated by methylxanthines (1, 5). Since the methylxanthines inhibit cyclic AMP phosphodiesterase activity (6, 7), and the amount of inhibition with theophylline is roughly proportional to the amount of lipolysis (2, 8), this has been assumed to be the mechanism by which these agents stimulate lipolysis. If this concept were correct, one might expect that the potencies as lipolytic agents of a series of substituted xanthines would parallel their potencies as inhibitors of phosphodiesterase activity. At least such a relationship should exist provided that the phosphodiesterase activity which was measured was related to the pool of cyclic AMP affecting lipolysis and that the effect of the inhibitors on this relevant phosphodiesterase activity was not distorted by such factors as permeability, binding, and metabolism of the substances. The purpose of the present study was to examine in adipose tissue a number of xanthine derivatives for a possible correlation between their effects on lipolysis and on

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phosphodiesterase activity and thereby to establish, if possible, a model system which could be applied to other tissues and other responses affected by the methylxanthines.

EXPERIMENTAL PROCEDURE

Materials. Crude bacterial collagenase was purchased from Worthington Biochemical Corporation, and *Crotalus atrox* venom from Ross Allen's Reptile Institute. Glycerol kinase and glycerophosphate dehydrogenase were purchased from Boehringer Mannheim Corporation. Bovine serum albumin (Fraction V) used in the lipolysis experiments was obtained from Nutritional Biochemicals Corporation and dialyzed against three changes of distilled water prior to use (9). The albumin used in the phosphodiesterase assay was obtained from Sigma Chemical Company (crystallized and lyophilized, lot 108B-8170). Theophylline and caffeine were obtained from Merck and Company, theobromine from Eastman Organic Chemicals, and diazoxide from the Schering Corporation. All other experimental compounds were purchased from Aldrich Chemical Company or were custom-synthesized and kindly supplied by the G. D. Searle Company, as indicated in Table 1. Concentrations of the compounds were determined by weighing and were not corrected for possible hydration. Mild heating or addition of small amounts of sodium hydroxide was needed to dissolve some of the compounds. None of these solutions gave a precipitate when diluted 10-fold with water or Tris-HCl buffer (pH 7.5), and the ultraviolet absorption spectra indicated that the compounds remained in solution after dilution. However, it could not be similarly established that the compounds stayed in solution when diluted with the complete lipolysis or phosphodiesterase assay mixtures, since the large amounts of protein present interfered with the optical density measurements.

Assay procedures. Isolated fat cells were prepared as described by Rodbell (10) from the epididymal adipose tissue of fed, 140–160-g rats. Aliquots of the cell suspension containing 25–30 mg of cells were added to Krebs-Ringer-bicarbonate buffer (pH 7.4) which contained varying amounts of the experimental compound. The final incubation volume was 2.5 ml and contained 3.5% albumin. The mixtures were incubated for 60 min at 37° in a covered, shaking water bath which was continuously gassed with a mixture of 95% O_2 and 5% CO_2. The reaction was terminated by addition of 0.25 ml of 30% perchloric acid, and the glycerol concentration of the incubation medium was assayed enzymatically (11). Both basal and theophylline-stimulated glycerol release were linear for at least 80 min under these conditions. The lipolytic effect of theophylline was determined for each batch of cells.

An aliquot of the original cell suspension was washed twice with 0.04 M Tris-HCl (pH 7.5) and then resuspended in 5 volumes of the same buffer to which bovine serum albumin had been added to a concentration of either 0.2% or 8.7%. This suspension was homogenized by means of a motor-driven ground glass pestle in a conical ground glass homogenizer. Cyclic AMP phosphodiesterase activity in the homogenates was determined as described elsewhere (12), with slight modifications. Briefly, tritiated cyclic AMP was incubated for 30 min at 37° with an appropriate amount of the homogenate and an excess of *Crotalus atrox* venom (which contains a 5′-nucleotidase); the reaction was stopped by addition of 2 volumes of a solution of nonlabeled cyclic AMP (0.1 mM) and heated in a 95–100° water bath for 75 sec; the tritiated product was separated from residual substrate by means of Dowex 2 anion exchange resin chromatography; and the amount of product and remaining substrate were determined in a liquid scintillation spectrometer. The initial concentration of cyclic AMP in the reaction mixture was 1 μM, and no more than 35% of this was hydrolyzed in the absence of inhibitor. The final concentration of albumin was either 0.08% or 3.5%. The effect of theophylline on phosphodiesterase activity was tested for each batch of cells.

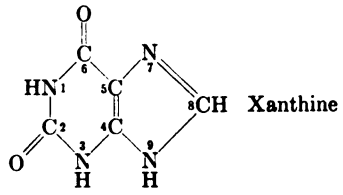
RESULTS

Effects of derivatives on lipolysis. Sixty-four compounds were tested at 0.05 and 0.50 mM for their lipolytic activities in the isolated fat cell system (Table 1). These two concentrations of theophylline produced about

TABLE 1

Lipolytic potencies relative to theophylline of 64 xanthine derivatives and related compounds

Lipolytic activities are expressed as the percentage of the glycerol released by the same concentrations of theophylline in the same experiment. The amounts of glycerol released by 0.05 and 0.50 mM theophylline were 1.14 ± 0.64 (SD) and 6.33 ± 1.44 μmoles/100 mg, dry weight, per hour, respectively (17 experiments). Compounds 1–56 are xanthine derivatives substituted at positions 1, 3, and 7, 8, or 9 as indicated. Compounds 58–64 are various uracil, purine, or benzothiadiazide derivatives. No. 57 is xanthine.



Xanthine

Compound	Position 1	Position 3	Positions 7, 8, 9	Lipolytic activity relative to theophylline	
				0.05 mM	0.50 mM
				%	%
1	Methyl	Methyl		100	100
2	Methyl	Methyl	(7) Methyl	16	47
3[a]	Methyl	Methyl	(7) Chloromethylene	91	74
4[a]	Methyl	Methyl	(7) Hydroxymethylene	113	85
5[b]	Methyl	Methyl	(7) 2-Chloroethyl	118	124
6[b]	Methyl	Methyl	(7) 2-Hydroxyethyl	<1	28
7[b]	Methyl	Methyl	(7) 2,3-Dihydroxyethyl	4	4
8[a]	Methyl	Methyl	(7) 2-Cyanoethyl	69	35
9[b]	Methyl	Methyl	(7) Ethyl acetate	7	<1
10[b]	Methyl	Methyl	(7) Piperadylmethylene	59	80
11[b]	Methyl	Methyl	(7) Morpholinylmethylene	92	82
12[a]	Methyl	Methyl	(7) D-Glucosyl	<1	<1
13[b]	Methyl	Methyl	(7) *N,N'*-Dimethylenepiperazyltheophylline[c]	185	110
14[a]	Methyl	Methyl	(7) Methyl (8) Isoamyloxy	82	28
15[a]	Methyl	Methyl	(8) Bromo	<1	<1
16[b]	Methyl	Methyl	(8) Chloro	<1	<1
17[a]	Methyl	Methyl	(8) Mercapto	<1	10
18[a]	Methyl	Methyl	(8) Nitro	53	91
19[a]	Methyl	2-Hydroxyethyl		14	61
20[a]	Methyl	Butyl		633	168
21[a]	Methyl	Isobutyl		1936	191
22[a]	Methyl	2-Isobutenyl	(8) Methyl	289	98
23[a]	Methyl		(7) Methyl (8) Chloro	<1	3
24[a]	Ethyl	Ethyl		498	115
25[a]	Ethyl	Ethyl	(7) Methyl	257	113
26[a]	Ethyl	Ethyl	(7) Ethyl	436	115

[a] Obtained from the G. D. Searle Company.

[b] Purchased from Aldrich Chemical Company, Inc.

[c] This compound is 2 theophylline molecules bridged at their 7-positions by *N,N'*-dimethylenepiperazine.

TABLE 1 —*Continued*

Compound	Position 1	Position 3	Positions 7, 8, 9	Lipolytic activity relative to theophylline	
				0.05 mM	0.50 mM
				%	%
27[a]	Ethyl	Ethyl	(7) 2-Hydroxyethyl	9	<1
28[a]	Ethyl	Ethyl	(8) Bromo	<1	<1
29[a]	Ethyl	Propyl		553	131
30[a]	Ethyl	Propyl	(9) Methyl	3	<1
31[a]	Ethyl	Butyl		593	170
32[a, d]	Ethyl	2-Isobutenyl		250	108
33[a]	Ethyl	2,3-Dibromopropyl	(8) Methyl	359	121
34[a]	Ethyl	2-Dimethylaminoethyl	(8) Benzyl	92	84
35[a]	Ethyl	Cyclohexyl		410	92
36[a]	Ethyl	2-Phenylethyl		220	21
37[a]	Ethyl	Ethyldimethylpropyl-ammonium bromide		3	17
38[a]	Ethyl	Ethyldimethylpropyl-ammonium bromide	(8) Methyl	<1	3
39[a]	2-Hydroxyethyl	Methyl		18	36
40[a]	Propyl	Propyl		410	106
41[a]	Propyl	Propyl	(7) Methyl	209	108
42[a]	Allyl	Allyl		246	110
43[a]	Allyl	Allyl	(8) Methyl	262	77
44[a]	2-Propynyl	Ethyl	(7) Ethyl	413	167
45[a]	Butyl	Butyl		519	90
46[a]	Pentyl	Ethyl		482	78
47[a]	Benzyl	Benzyl		59	69
48		Methyl	(7) Methyl	<1	17
49[a]		2-Hydroxyethyl		16	65
50[a]		2-Hydroxyethyl	(8) Ethyl	54	3
51[a]		2-Hydroxyethyl	(7) 2-Hydroxyethyl	83	100
52[a]		Propyl	(7) Butyl	83	89
53[a]		Propyl	(7) Ethyl-*N*-dimethyl	13	7
54[a]		Propyl	(7) Ethyldimethylpropylammonium bromide	11	<1
55[a]		Allyl		21	31
56[b]			(8) Bromo (9) D-Ribosyl	4	<1
57[b]				<1	<1
58[a]	1-Allyl-6-amino-3-ethyluracil (aminometramide)			8	9
59[b]	2-Amino-6-hydroxy-8-mercaptopurine			13	3
60[b]	2-Mercapto-6,8-purinediol			2	2
61[b]	*N*,*N*-Bis(2-hydroxyethyl)-6-aminopurine			1	1
62[b]	Purine-6-thioacetic acid			1	1
63[b]	1-Benzyl-6-oxy-9-D-ribosylpurine			1	1
64	7-Chloro-3-methyl-2*H*-1,2,4-benzothiadiozine 1,1-dioxide (diazoxide)			12	3

[d] May be isomer where positions 1 and 3 are reversed.

10% and 60%, respectively, of the maximal response which could be elicited by theophylline. At the lower concentration, 0.05 mM, the most active compound, 1-methyl-3-isobutylxanthine, caused 19 times more glycerol release than 0.5 mM theophylline; 12 other

compounds produced several times greater lipolysis than theophylline at this concentration.

Some of the compounds, including Nos. 36 and 50 (Table 1), were considerably less lipolytic at 0.5 mM than at 0.05 mM, indicating that at higher concentrations these compounds inhibited lipolysis. In general, the compounds are arranged in Table 1 according to increasing size of the substituent on positions 1, 3, and 7, respectively. Several exceptions to this system were made in order to keep homologous series of compounds together.

Effects of derivatives on phosphodiesterase activity. Nine compounds of widely varying apparent lipolytic potencies were then tested over a 20-fold range of concentrations as lipolytic agents and as cyclic AMP phosphodiesterase inhibitors (Figs. 1 and 2). There was good general agreement between the orders of relative potencies in the two systems over the entire range of concentrations. One compound, 1,3-dimethyl-8-mercaptoxanthine (No. 17), was more effective as an inhibitor of phosphodiesterase activity when the concentration of albumin in the assay medium was lowered (Fig. 3). A similar, though quantitatively smaller, effect was seen with 1,3-diethylxanthine (No. 24). No appreciable difference between the apparent potencies at the high and low albumin concentrations was noted for any of the other compounds.

Substantial increases in lipolysis were associated with only partial inhibition of phosphodiesterase activity. For instance, concentrations of any of the compounds which inhibited phosphodiesterase activity 20% produced easily detectable increases in lipolysis (Figs. 1 and 2).

None of the compounds interfered with the assay for glycerol or with the effectiveness of the venom (5′-nucleotidase) used in the assay for phosphodiesterase activity. The amounts of sodium hydroxide added to aid in solubilizing some of the compounds did not change either the pH of the assay mixtures or the rate of basal glycerol release from the isolated cells. Glycerol release in the presence of the nine compounds listed in Figs. 1 and 2 was virtually linear for at least 80 min, as illustrated in Fig. 4 by the most potent and one of the least potent of these compounds.

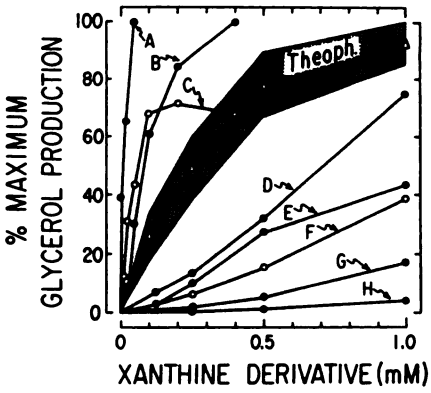


FIG. 1. *Stimulation of lipolysis in isolated fat cells by xanthine derivatives and other compounds*

Cells were incubated with the experimental compounds, and lipolysis was measured by determining the glycerol released into the incubation medium. The results are expressed as the percentage of the maximal response produced by theophylline in the same experiment. The amounts of glycerol released with no theophylline and 2 mM theophylline (taken to be maximal) were 0.42 ± 0.13 (SD) and 7.30 ± 1.84 μmoles/hr/100 mg, dry weight, respectively (14 experiments). The albumin concentration was 3.5%. The points on the theophylline curve are the mean values of 14 experiments ± standard deviation (shaded area). The other curves represent single experiments done concurrently with those shown in Fig. 2. The compounds tested were: *A*, 1-methyl-3-isobutylxanthine (No. 21 in Table 1); *B*, 1,3-diethylxanthine (No. 24); *C*, 1-pentyl-3-ethylxanthine (No. 46); *D*, 1,3,7-trimethylxanthine [caffeine (No. 2)]; *E*, 3,7-dimethylxanthine [theobromine (No. 48)]; *F*, 1-allyl-6-amino-3-ethyluracil [aminometramide (No. 58)]; *G*, 3-propyl-7-ethyl-*N*-dimethylxanthine (No. 53); *H*, 1,3-dimethyl-8-mercaptoxanthine (No. 17).

DISCUSSION

The close agreement between the potencies as lipolytic agents and as cyclic AMP phosphodiesterase inhibitors of this series of xanthine derivatives strongly indicates that their lipolytic activity is mediated via their effects on phosphodiesterase. The data also suggest that only partial inhibition of phosphodiesterase activity is necessary to raise the effective level of cyclic AMP in the cell.

Although no systematic determination of

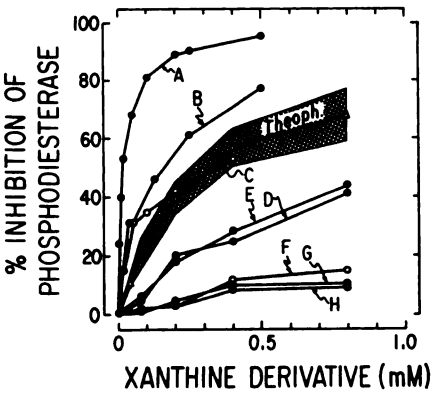


FIG. 2. *Inhibition of cyclic AMP phosphodiesterase by xanthine derivatives and other compounds*

The phosphodiesterase activity in homogenates of the same cell suspensions used to obtain the results in Fig. 1 was determined by measuring the amount of radioactive product formed from tritiated cyclic AMP (1 μM) during a 30-min incubation at 37°. Less than 35% of the initial substrate was hydrolyzed in the control (uninhibited) tubes. The albumin concentration was 3.5%. The theophylline curve was taken from the mean values of six experiments ± standard deviation (shaded area), and the other curves were taken from single experiments done concurrently with those shown in Fig. 1. Compounds *A* through *H* are identified in the legend to Fig. 1.

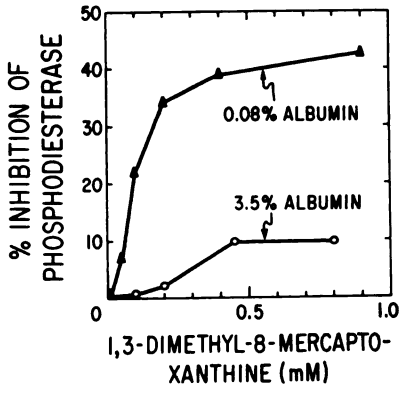


FIG. 3. *Effects of albumin concentration on phosphodiesterase-inhibitory activity of 1,3-dimethyl-8-mercaptoxanthine (No. 17 in Table 1)*

Conditions were as described in Fig. 2.

structure-activity relationships was attempted, several general observations can be made. The most active derivatives were those which contained small nonpolar groups on both positions 1 and 3 (e.g., ethyl, propyl, isobutyl). Substitutions of a halide group on position 8 caused the compound to be inactive. Similarly, the three compounds having a substitution on position 9 were inactive. In general, substitutions made on position 7 caused either no change in the potency of the compound or a reduction in potency.

Several possible problems inherent in the interpretation of these studies must be considered. First, erroneous interpretations could arise if some of the compounds were bound to a component of the incubation medium. For example, 1,3-dimethyl-8-mercaptoxanthine was a better inhibitor of phosphodiesterase activity when the albumin concentration of the medium was lowered. Its lipolytic activity then may have been affected by its binding to albumin. Similarly, erroneous interpretations might be made if there were differences in abilities of the compounds to enter the unbroken cell. A compound having such difficulty might inhibit phosphodiesterase activity in homogenates of the cells but be unable to produce the expected response in the intact cell. However, this may not be a serious problem with most xanthine derivatives, since caffeine, at least, does cross cell membranes readily (13).

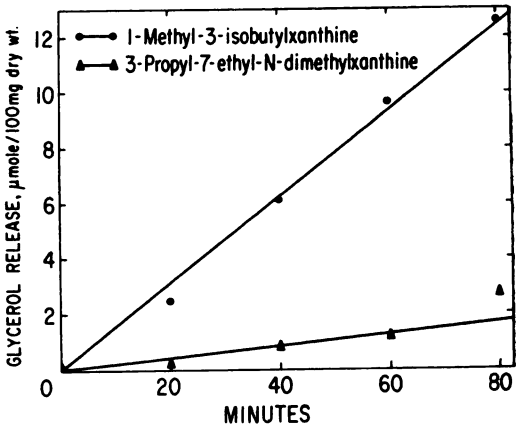


FIG. 4. *Time course for glycerol release from isolated fat cells in response to substituted xanthines*

The concentration of the experimental compounds was 0.5 mM. 1-Methyl-3-isobutylxanthine is No. 21 in Table 1, and 3-propyl-7-ethyl-*N*-dimethylxanthine is No. 53. Conditions and procedures were as described under EXPERIMENTAL PROCEDURE.

Other potential problems could arise if the compounds were metabolized by the cell type under study, or if they were only partially soluble in the assay mixtures. Any of these factors could have been responsible for the slight discrepancy between the lipolytic and phosphodiesterase-inhibitory potencies seen with two of the compounds, 1,3-diethylxanthine and 1-pentyl-3-ethylxanthine (*B* and *C* in Figs. 1 and 2). In addition, it should be recognized that the amount of phosphodiesterase inhibition occurring in a broken cell preparation may not be an accurate reflection of that occurring in the intact cell. Furthermore, especially in studies which would involve broken cell preparations from heterogeneous cell populations, a substantial amount of the phosphodiesterase activity measured may not be related to the cell type producing the physiological event of interest. Evidence exists that more than one form of phosphodiesterase activity is present within at least two different tissues (12, 14, 15), and unless all forms of the enzyme respond similarly, the effects of inhibitors on the relevant phosphodiesterase activity may be obscured in some preparations. Even when using a preparation from a homogeneous cell population, such as isolated fat cells, it is an assumption that most if not all of the phosphodiesterase activity being measured is, or responds similarly to, that which is related to lipolysis. Despite these difficulties in interpretation, the experimental approach of comparing the potencies of a series of xanthine derivatives as inhibitors of cyclic nucleotide phosphodiesterase activity with their potencies in producing a physiological response may be useful as an aid in clarifying the diverse pharmacological effects of these agents in other tissues.

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