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METHYLXANTHINE AND NON-XANTHINE PHOSPHODIESTERASE INHIBITORS

THEIR EFFECTS ON ADENOSINE UPTAKE AND THE LOW K_m CYCLIC AMP PHOSPHODIESTERASE IN INTACT RAT ADIPOCYTE

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Abstract—The effects of methylxanthines and non-xanthine phosphodiesterase-inhibitors on the low K_m cyclic AMP phosphodiesterase of intact rat adipocytes were studied. Methylxanthines and papavarine stimulated rather than inhibited the enzyme when intact adipocytes were incubated in their presence. The effect of papavarine was not abolished by adenosine deaminase and was enhanced by adenosine. On the other hand, the effect of xanthine inhibitors and adenosine do not enhance each other.

The difference in behaviour of these inhibitors could not be explained by their effects on adenosine uptake at the concentrations studied. Both agents inhibited adenosine uptake when measured after 15 sec and 10 min, with methylisobutylxanthine (MIX) having a greater inhibitory effect than papavarine only if uptake was measured after 15 sec.

Effects similar to that of adenosine with the inhibitors on phosphodiesterase were obtained with insulin, which has been shown to act through a similar or related mechanism to that of adenosine. This was not the case with lipolytic agents whose effects were not potentiated by either MIX or papavarine. Under certain conditions the degree of stimulation of the enzyme was in fact decreased. Thus lipolytic and antilipolytic agents probably stimulated phosphodiesterase through distinct mechanisms.

The relative rates of formation and degradation of cyclic nucleotides are assumed to determine the steady-state levels of these nucleotides in most cells. The inactivation of cyclic AMP in adipose and other tissues involved the hydrolysis of the 3′ phosphate bond through the action of cyclic nucleotide phosphodiesterase [1]. Methylxanthines which inhibit phosphodiesterase activity [2, 3] have been shown to elevate cyclic AMP levels in adipose tissue and isolated fat cells [4, 5]. If the basal turnover of the cyclic nucleotide is rapid enough for inhibition of phosphodiesterase to cause increases in cyclic nucleotide levels, then phosphodiesterase inhibition alone should mimic the action of a hormone if cyclic nucleotides are involved in the response to the hormone [6]. Methylxanthines have been shown to be potent stimulators of the lipolytic process [7, 8] and it has been assumed that the lipolytic activity is secondary to the inhibition of the phosphodiesterase [3, 4]. There is, however, some evidence that the lipolytic response to theophylline could involve factors other than the inhibition of phosphodiesterase [9]. Under certain conditions, the phosphodiesterase inhibition did not correlate with the stimulation of lipolysis or increase in tissue concentrations of cyclic AMP [10]. Other strong inhibitors of phosphodiesterase, such as papavarine and dipyridamole [11–14] were also found not to produce lipolysis [13, 15, 16]. It has also been reported that the partially purified phosphodiesterase (P-2 fraction) was activated when isolated from methylxanthine-treated fat cells and hepatocytes [17–19], in contrast to findings from studies on the enzyme in cell homogenates and crude enzyme preparations [10, 15, 20]. As such differences have also been observed for the effects of adenosine on cyclic AMP phosphodiesterase from whole cells and that of homogenates [21] it was felt that the effects of phosphodiesterase inhibitors should be reexamined with whole cells, especially when there appeared to be a lack of correlation between lipolytic activity in intact fat cells and phosphodiesterase inhibition in homogenates [10].

In the present study, various effects of theophylline, 1-methyl-3-isobutylxanthine (MIX), papavarine and dipyridamole on the low K_m cyclic AMP phosphodiesterase of intact rat adipocytes were examined. Since the effects of the methylxanthines on the enzyme were related to its competition with adenosine for its receptor [22–25] whilst those of papavarine and dipyridamole, on the uptake of adenosine [26], the effects of these agents on adenosine uptake were also investigated.

MATERIALS AND METHODS

Most chemicals including bovine serum albumin (fraction V), Hepes, adenosine, theophylline, MIX, papavarine, dipyridamole, collagenase (type II), cyclic AMP, dibutyryl cyclic AMP, 5′ nucleotidase (from crotalus adamanteus venom 77 units/mg protein) and adenosine deaminase, were obtained from Sigma Chemical Co., St. Louis, MO. Dinonylphthalate was obtained from B.D.H., Poole, U.K.

Cyclic [^{3}H]AMP (37.7 Ci/nmole) and [2-^{3}H]adenosine (22.3 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, U.K.

Isolated fat cells were prepared by the collagenase method of Rodbell [27] from the epididymal adipose tissue of male Wistar rats (180–200 g), in the presence of adenosine deaminase as described in [28]. Similar conditions were also used to carry out experiments on phosphodiesterase activity.

Uptake of adenosine was studied with the aid of [2-^{3}H]adenosine with a final sp. act. of 1 Ci/10 moles. The adenosine and fat cell concentrations used were similar to those for the phosphodiesterase experiments. Usually, 2-ml aliquots of cells were distributed into plastic vials containing adenosine with or without other compounds in 0.5 ml buffer solution. Unless otherwise indicated, quadruplicate 0.2-ml aliquots were immediately withdrawn and the fat cells separated quickly from the medium by centrifugation for 10 sec with 0.1 ml dinonylphthalate in 0.4-ml microfuge tubes in a Beckman 152 microfuge. The tubes were cut at the dinonylphthalate interface and the portion with the fat cells allowed to fall into 10 ml toluene-based scintillant. Radioactivity was measured after standing for 30 min during which the fat cell pellet, with some agitation, completely disintegrated in the toluene. This procedure was repeated at the end of the experiment after incubating the cell suspension in a shaking-water bath for 10 min at 37°. Non-specific binding was found to be 2% or less and was therefore not corrected for.

Results are reported as the mean ± S.E.M. of duplicate incubations, measured in triplicate with at least three different batches of cells being used.

RESULTS AND DISCUSSION

Effects of adenosine on the action of methylxanthines and papavarine on phosphodiesterase

Table 1 shows the effects of MIX and theophylline on the low K_m cyclic AMP phosphodiesterase on intact rat adipocytes, incubated in the presence and absence of adenosine. Both compounds had a stimulatory rather than an inhibitory effect on the enzyme. These results are consistent with the observations of Pawlson *et al.* [17] who studied the effects of theophylline (0.5 mM) on adipocytes and more recently, Heyworth *et al.* [19] with MIX (1 mM) on hepatocytes. These agents did not potentiate the stimulatory effect of adenosine which was potentiated by papavarine (Table 2). Papavarine also increased the phosphodiesterase activity of fat cells in the absence of adenosine and the presence of adenosine deaminase. This is contrary to the belief that the effect of papavarine was primarily due to an increase in the concentration of adenosine in the medium resulting from its inhibition of adenosine uptake into the cell [22]. To account for this discrepancy, the effect of inosine was studied, in case it had an effect on papavarine. Inosine had no effect on phosphodiesterase activity, neither did it enhance the stimulatory effect of papavarine on the enzyme (Table 2).

Effects of methylxanthines, papavarine and dipyridamole on the uptake of adenosine by fat cells

The effects of MIX and papavarine on adenosine uptake was investigated as they are known to affect this process differently [26]. Since the study was in relation to their effects on phosphodiesterase activity, experimental conditions similar to those used for such studies were employed. Uptake studies were carried out over 15 sec and over 10 min. The shortest period possible was selected as this would largely reflect the amount of adenosine bound to the cells. Ten minutes was the period over which the effect of adenosine on phosphodiesterase activity had been investigated. Figure 1 shows the uptake of adenosine at increasing concentrations. The uptake of adenosine by the fat cells was linearly related to adenosine concentration between 2 and 20 μM. This is similar to that observed in red-cell ghosts [29] and cerebral cortex slices of guinea pig [30]. Figure 2

Table 1. Effects of methylxanthines and adenosine on phosphodiesterase activity

Additions	Phosphodiesterase activity (% of control)
Experiment A	
ADA* 1 U/ml (control)	100
Theophylline (10^{-3} M)+ ADA	119.9 ± 1.1
MIX† (10^{-3} M)+ ADA	124.1 ± 4.0
Adenosine (10^{-5} M)	135.8 ± 2.6
Theophylline + adenosine	139.7 ± 3.1
MIX + adenosine	137.5 ± 5.5
Experiment B	
None	100
MIX (10^{-3} M)	130.5 ± 2.2
Adenosine (10^{-5} M)	124.7 ± 1.5
MIX + adenosine	134.0 ± 2.9

Cells were incubated with or without the addition of various agents for 10 min, washed twice with sucrose–Tris pH 7.5 and particulate fraction P-2 prepared as described in [28].

* Adenosine deaminase, 1 U/ml.

† Methylisobutylxanthine.

Table 2. Effects of papavarine and adenosine on phosphodiesterase activity

Additions	Phosphodiesterase activity (% of control)
Experiment A	
ADA* 1 U/ml	100
Papavarine (2×10^{-4} M)+ ADA	131.7 ± 2.9
None	100
Papavarine (2×10^{-4} M)	135.5 ± 2.0
Adenosine (10^{-5} M)	136.5 ± 4.0
Papavarine + adenosine	169.0 ± 3.1
Experiment B	
Adenosine deaminase (1 U/ml)	100
Inosine (10^{-5} M)+ ADA	97.7 ± 3.3
Adenosine (10^{-5} M)	125.8 ± 3.5
Papavarine (2×10^{-4} M)	131.6 ± 2.8
Inosine + papavarine	134.5 ± 2.9
Adenosine + papavarine	155.9 ± 4.9

Experimental conditions and determination of phosphodiesterase activity are as described for Table 1.
* Adenosine deaminase, 1 U/ml.

shows that adenosine uptake was inhibited by both MIX and papavarine. Methylisobutylxanthine had a greater inhibitory effect than papavarine when uptake was measured after 15 sec. This is consistent with the effect of MIX on adenosine binding to its receptor. The data in Table 3 provides a comparison of the effects of MIX, theophylline, papavarine and dipridamole at the respective concentrations normally used for the study of these inhibitors, i.e. 1 mM for the methylxanthines and 200 μM for papavarine and dipyridamole. At both 200-μM and 1-mM and 1-μM concentrations, MIX had a greater effect on adenosine binding (uptake after 15 sec) than all the other compounds tested. On the other hand, at a concentration of 200 μM, papavarine and MIX were potent in inhibiting 50% of the adenosine uptake over 10 min. At the higher concentration of 1 mM, the effect of papavarine was, however, significantly greater than MIX and theophylline. Thus the effect of papavarine can be assumed to be due to its effect on adenosine uptake only at high concentrations.

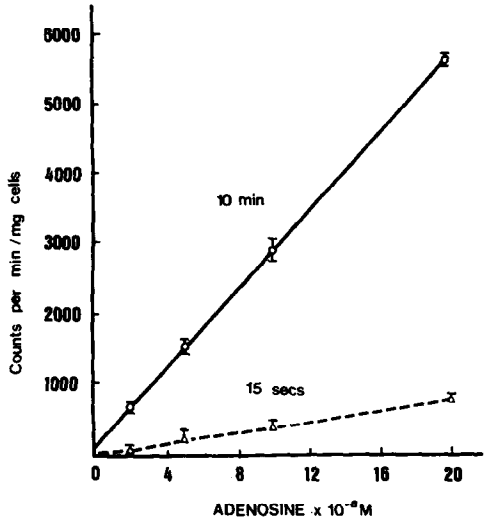

Fig. 1. Effect of adenosine concentration on the uptake of labelled adenosine. Fat cells (20 mg/ml) were incubated with various adenosine concentration of sp. act. 1 Ci/10 mmoles in 2.5- and 0.2-ml aliquots withdrawn after 15-sec or 10-min incubations for determination of labelled adenosine uptake as described in Materials and Methods.

Effects of insulin and some lipolytic agents on the action of 1-methyl-3-isobutylxanthine and papavarine on phosphodiesterase

The effects of MIX and papavarine on the stimulation of phosphodiesterase activity by insulin were studied as there appeared to be some similarity between the action of insulin and that of adenosine on phosphodiesterase [28]. The effects were studied

Table 3. Comparison of the effects of methylxanthines, papavarine and dipyridamole on uptake of labelled adenosine at concentrations normally used in metabolic studies

Additions	Adenosine uptake (% of control) 2×10^{-4} M	10^{-3} M
15-sec incubation		
None (control)	100	100
Papavarine	63.6 ± 0.5	24.0 ± 0.4
Dipyridamole	83.6 ± 0.3	—
Methylisobutylxanthine	25.8 ± 0.1	19.0 ± 0.5
Theophylline	68.5 ± 0.2	31.0 ± 0.4
10-min incubation		
None (control)	100	100
Papavarine	44.5 ± 0.2	3.6 ± 0.4
Dipyridamole	52.7 ± 0.3	—
Methylisobutylxanthine	43.9 ± 0.5	15.5 ± 0.4
Theophylline	85.2 ± 0.4	45.8 ± 0.3

Fat cells were exposed to labelled adenosine (1 Ci/10 mmoles) in the absence and presence of various inhibitors and the uptake of adenosine determined as described in the legends to Fig. 1.

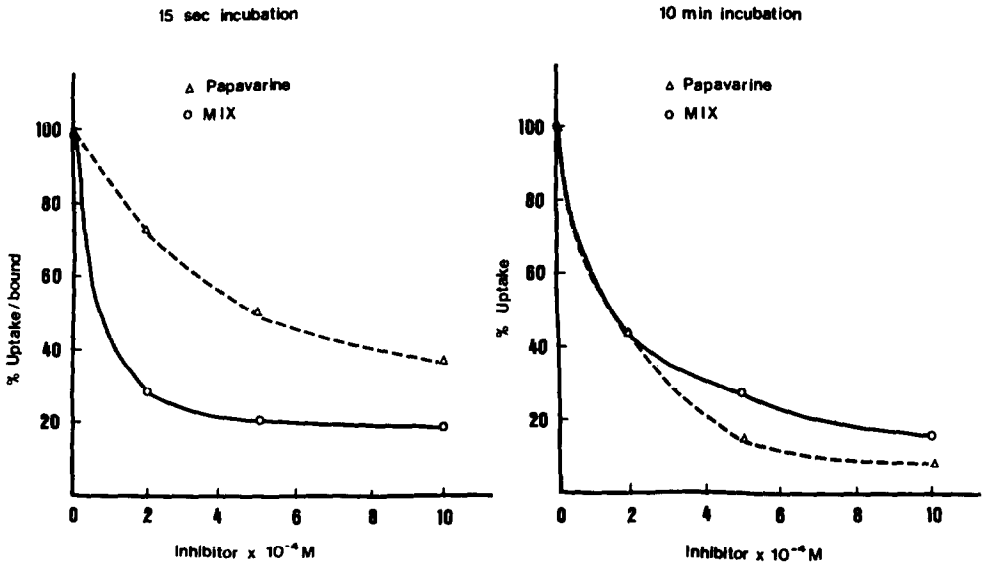

Fig. 2. Effect of 1-methyl-3-isobutylxanthine (MIX) and papavarine on uptake of labelled adenosine. Fat cells (18 mg/ml) were incubated for 15 sec or 10 min with 10^{-5} M adenosine (sp. act. 1 Ci/10 mmoles) in the presence of various concentrations of papavarine or MIX. The uptake of labelled adenosine was determined as for experiments in Fig. 1.

Table 4. Effects of methylisobutylxanthine and papavarine on phosphodiesterase activity in the presence of insulin

Additions	Phosphodiesterase activity (% of control)
ADA* (1 U/ml)	100
MIX (10^{-3} M) + ADA	132.9 ± 5.8
Insulin (100 μU/ml)+ ADA	147.7 ± 4.2
MIX + insulin + ADA	153.2 ± 2.2
Papavarine (2×10^{-4} M) + ADA	141.2 ± 1.9
Papavarine + insulin + ADA	163.4 ± 2.6

Experimental conditions and determination of phosphodiesterase activity are as described for Table 1.
* Adenosine deaminase 1 U/ml.
† Methylisobutylxanthine.

Table 5. Effect of methylisobutylxanthine on phosphodiesterase activity in the presence of isoproterenol or dibutyryl cyclic AMP

Additions	Phosphodiesterase activity (% of control)
Experiment A	
ADA* (1 U/ml)	100
Isoproterenol (10^{-6} M) + ADA	124.1 ± 2.0
MIX† (10^{-3} M) + ADA	132.4 ± 3.5
MIX + isoproterenol + ADA	120.7 ± 3.3
Experiment B	
ADA (1 U/ml)	100
Dibutyryl cyclic AMP (10^{-3} M) + ADA	133.7 ± 1.3
MIX (10^{-3} M) + ADA	150.5 ± 2.6
MIX + dibutyryl cyclic AMP + ADA	136.4 ± 1.8

Experimental conditions and determination of phosphodiesterase activity are as described for Table 1.
* Adenosine deaminase 1 U/ml.
† Methylisobutylxanthine.

in the presence of adenosine deaminase (1 unit/ml) to eliminate the effects due to adenosine. The results are shown in Table 4. The effects of insulin was enhanced by papavarine in a manner which was very similar to that observed with adenosine (Table 1).

Isoprenaline and glucagon also have been shown to stimulate the phosphodiesterase of adipocytes and hepatocytes [28, 31]. Unlike adenosine and insulin, these are lipolytic agents known to raise cyclic AMP levels in the cell. It would be interesting to see what effects MIX and papavarine would have on their action especially when the mechanism of action of lipolytic and antilipolytic agents on phosphodiesterase is still not clear [32, 28]. The effects of isoprenaline, epinephrine and dibutyryl cyclic AMP were investigated and the results presented in Tables 5 and 6. Like insulin and adenosine, these agents also stimulated phosphodiesterase activity and their effects were not potentiated by MIX (Table 5). Papavarine, however, did not enhance their effects as it did for adenosine and insulin (Tables 2, 4 and 6). On the other hand, if the stimulation of the phosphodiesterase activity due to the lipolytic agent was greater than that due to either of these two compounds, the presence of MIX or papavarine, together with the lipolytic agent in the incubating medium, resulted in the lowering of the degree of stimulation of the enzyme.

This phenomenon was also observed when the stimulation due to MIX or papavarine alone was greater than that due to the lipolytic agent (Tables 5 and 6). Similarly when MIX was added to the cells

Table 6. Effect of papavarine on phosphodiesterase activity in the presence of insulin, isoproterenol, epinephrine, adenosine and dibutyryl cyclic AMP

Additions	Phosphodiesterase activity (% of control)
Experiment A	
ADA* (1 U/ml)	100
Papavarine (2×10^{-4} M) + ADA	130.3 ± 1.3
Epinephrine (10^{-6} M) + ADA	142.4 ± 1.1
Papavarine + epinephrine + ADA	129.1 ± 4.4
Adenosine (10^{-5} M)	137.7 ± 1.4†
Papavarine + adenosine	149.0 ± 0.7†
Experiment B	
None	100
Papavarine (2×10^{-4} M)	133.3 ± 1.7
Dibutyryl cyclic AMP (10^{-3} M)	122.7 ± 5.8
Papavarine + dibutyryl cyclic AMP	115.9 ± 4.4
Adenosine (10^{-5} M)	140.0 ± 2.4†
Papavarine + adenosine	155.5 ± 1.5†
Experiment C	
ADA (1 U/ml)	100
Papavarine (2×10^{-4} M) + ADA	135.9 ± 2.6
Isoproterenol (10^{-6} M) + ADA	154.7 ± 4.9
Papavarine + isoproterenol + ADA	150.2 ± 3.8
Insulin (100 μU/ml) + ADA	142.5 ± 2.2†
Papavarine + insulin + ADA	160.3 ± 3.5†

Experimental conditions and determination of phosphodiesterase activity are as described for Table 1.

* Adenosine deaminase, 1 U/ml.

† $P < 0.05$ for effect of papavarine.

Table 7. Effects of papavarine and dipyridamole on phosphodiesterase activity in the presence of methylisobutylxanthine

Additions	Phosphodiesterase activity (% of control)
ADA* (1 U/ml)	100
Papavarine (2×10^{-4} M) + ADA	138.6 ± 3.1
Dipyridamole (2×10^{-4} M) + ADA	139.2 ± 3.0
MIX† (10^{-3} M) + ADA	133.7 ± 2.3
Papavarine + MIX + ADA	130.4 ± 1.7
Dipyridamole + MIX + ADA	129.9 ± 2.1

Experimental conditions of phosphodiesterase activity are as described for Table 1.

* Adenosine deaminase, 1 U/ml.

† Methylisobutylxanthine.

along with either papavarine or dipyridamole the effect was never greater than the effect of the most active agent, but was in fact usually somewhat less (Table 7).

Thus the results of the above experiments, in particular the effects of papavarine, seem to indicate that the mechanisms through which lipolytic and antilipolytic agents exert their influence on the low K_m cyclic AMP phosphodiesterase were different. A possible complex interplay at the plasma-membrane receptor level has been suggested by Heyworth *et al.* [19] when MIX and dibutyryl cyclic AMP failed to mimic glucagon in eliciting a synergistic increase in the enzyme activity, on subsequent exposure of rat hepatocytes to insulin. In the present study (Table 4) MIX also did not mimic isoprenaline [28] in potentiating the effect of insulin on rat adipocytes. On the other hand, insulin effects were potentiated by papavarine (Table 6), a phosphodiesterase inhibitor known not to raise cyclic AMP [20]. Similarly Heyworth *et al.* [19, 33] have also shown that cholera toxin and GTP activate cyclic AMP phosphodiesterase and potentiate the effect of insulin. The possible involvement of a guanine-nucleotide regulatory protein in mediating these effects were therefore suggested. We found, however, that although GTP also stimulated fat cell cyclic AMP phosphodiesterase it did not potentiate the effect of insulin (unpublished data).

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