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Interactions between *Alpha*-Adrenergic Agents, Prostaglandin E_1, Nicotinic Acid, and Adenosine in Regulation of Lipolysis in Hamster Epididymal Adipocytes

RICHARD J. SCHIMMEL, KATHRYN K. MCMAHON, AND ROSALIND SERIO

Department of Physiology, College of Medicine and Dentistry of New Jersey, New Jersey School of Osteopathic Medicine, Piscataway, New Jersey 08854

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SUMMARY

SCHIMMEL, R. J., K. K. MCMAHON, AND R. SERIO. Interactions between *alpha*-adrenergic agents, prostaglandin E_1, nicotinic acid, and adenosine in regulation of lipolysis in hamster epididymal adipocytes. *Mol. Pharmacol.* 19:248–255 (1981).

The antilipolytic activity of the *alpha*-adrenergic agent clonidine was studied using fat cells from the epididymal fat bodies of golden hamsters. Lipolysis activated in a relatively dilute suspension of adipocytes (<30,000 cells/ml) with either 3-isobutyl-1-methylxanthine or isoproterenol was inhibited by clonidine. At cell densities >300,000 cells/ml, only 3-isobutyl-1-methylxanthine-accelerated lipolysis was inhibited by clonidine; isoproterenol-activated lipolysis was not. The antilipolytic activity of clonidine toward isoproterenol-activated lipolysis was also lost when adipocytes (<30,000 cells/ml) were incubated in media previously used for cell incubation. These findings suggested the appearance of a substance in the incubation medium which inhibited the antilipolytic activity of clonidine. The antilipolytic activity of clonidine was dramatically enhanced by addition of adenosine deaminase or theophylline to incubation media, suggesting that the substance is adenosine. Clonidine inhibited cyclic AMP accumulation to a greater extent in the presence of either adenosine deaminase or theophylline. The antilipolytic activity of prostaglandin E_1 and nicotinic acid, but not insulin, was also enhanced by the presence of adenosine deaminase. The release of adenosine from adipocytes in which the nucleotide pool was labeled by incubation with either [^{14}C]- or [^{3}H]adenine was readily detected after 5 min of incubation and was maximal after 30 min. From these data we conclude that the sensitivity of fat cells to the antilipolytic effects of clonidine, prostaglandin E_1, and nicotinic acid is strongly influenced by the presence of adenosine produced by the incubated cells and that optimal antilipolytic activity of these agents is seen only in the absence of adenosine.

INTRODUCTION

Persuasive evidence from this and other laboratories has demonstrated a prominent *alpha*-adrenergic sensitivity of hamster adipocytes (1–6). *Alpha*-receptor activation has been shown to antagonize activation of triglyceride hydrolysis and accumulation of cellular cyclic AMP, to decrease the activity of the cyclic AMP-dependent protein kinase, and to inhibit adenylate cyclase (2–6). Conversely, *alpha*-receptor blockade accelerates lipolysis, promotes accumulation of cyclic AMP, and activates the cyclic AMP-dependent protein kinase (1, 3, 5).

The cellular mechanism of action of *alpha*-receptor agonists on hamster adipocytes is not known with certainty. *Alpha*-adrenergic suppression of cyclic AMP levels results most likely from inhibition of adenylate cyclase rather than from activation of cyclic AMP phosphodiesterase (5, 6). However, a role of changes in cyclic AMP levels in mediating the antilipolytic effect of *alpha* agents has not been rigorously established. Although inhibition of cyclic AMP accumulation and activity of the cyclic AMP-dependent protein kinase are frequently observed to accompany *alpha*-adrenergic suppression of lipolysis (2–4), *alpha*-adrenergic inhibition of lipolysis has been found to be dissociated from changes in cell cyclic AMP levels under some conditions (2, 4). These observations have raised the possibility that *alpha*-adrenergic agents inhibit lipolysis by two mechanisms, one dependent upon cyclic AMP, the other independent of cyclic AMP (2).

Results of previous experiments from our laboratory

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suggested that the antilipolytic activity of some *alpha* site-selective agonists was influenced by the density of the incubated adipocyte (4). This observation raised the possibility of the existence of an endogenously produced substance which influenced the biological activity of *alpha*-adrenergic agents. The present study demonstrates that the antilipolytic activity of *alpha*-adrenergic agents is strongly influenced by the presence of adenosine which is elaborated by the cells during incubation.

MATERIALS AND METHODS

Chemicals. Collagenase (Type I) was obtained from Worthington Biochemical Corporation, Freehold, N. J.; isoproterenol, phenylephrine, theophylline, cyclic AMP, adenosine, inosine, hypoxanthine, ATP, AMP, and bovine serum albumin, Lot 97C-0411 (Fraction V) from Sigma Chemical Company, St. Louis, Mo.; adenosine deaminase from Sigma Chemical Company and from Boehringer Mannheim, New York, N. Y., nicotinic acid from Nutritional Biochemicals, Cleveland, Ohio; 3-isobutyl-1-methylxanthine from Aldrich Chemical Company, Milwaukee, Wisc.; methoxamine from Burroughs-Wellcome Corporation, Detroit, Mich.; insulin from Eli Lilly and Company, Indianapolis, Ind. Prostaglandin E_1 was a generous gift from The Upjohn Company, Kalamazoo, Mich.; clonidine was generously provided by Boehringer Ingelheim. [^{3}H]Adenine was obtained from Amersham/Searle Corporation, Arlington Heights, Ill.; [^{14}C]Adenine was obtained from New England Nuclear Corporation, Boston, Mass. All other chemicals were reagent grade of the best quality available.

Preparation and incubation of adipocytes. Golden hamsters weighing between 100 and 120 g were purchased from Charles River Laboratories, Lakeview, N. J., and fed Purina laboratory chow and tap water *ad libitum* for at least 1 week before use. They were killed by cervical dislocation, the epididymal fat bodies were removed, and adipocytes were prepared as previously described (2–5). For experiments in which the effects of cell density were studied (Tables 1 and 2), the following procedure was followed. After the last wash, an aliquot of cells was counted in a hemocytometer and the volume of the cell suspension was adjusted to yield a concentration of either 600,000 cells/ml or 1,000,000 cells/ml depending upon the cell density desired in the incubation. Aliquots of 50 μl from 600,000 cells/ml or of 200 μl from 1,000,000 cells/ml were transferred to plastic incubation vessels containing Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml (pH 7.4), and various hormones and other agents to yield cell densities of either 30,000 cells/ml or 300,000 cells/ml. For all other experiments, the volume of the cell suspension was adjusted to yield a final cell density of approximately 200,000 cells/ml. Cells were incubated with shaking at 37° under an atmosphere of 95% O_2 and 5% CO_2.

All experiments reported herein are the average of at least three replicate experiments performed on separate days. Each experiment in turn involved two or three replicate incubations for each condition employed.

Measurement of cyclic AMP and glycerol. Cyclic AMP accumulation in cells plus medium was assayed after trichloroacetic acid extraction by a modification of the protein kinase assay of Gilman (7). The glycerol released into the medium was measured fluorometrically by the enzymatic method of Wieland (8). The glycerol and cyclic AMP standards were prepared in incubation media containing bovine serum albumin and the drugs and hormones present in that particular experiment.

Determination of adenosine release. Adipose tissue was minced with scissors and incubated in 5 ml of Krebs-Ringer bicarbonate buffer containing albumin, 40 mg/ml, and [8-^{14}C]adenine, 2 μCi/ml (specific activity 42.3 mCi/mmole), or [8-^{3}H]adenine, 2 μCi/ml (specific activity 24 Ci/mmole). Collagenase, 1.4 mg/ml, was added after 55 min and the incubation was continued for an additional 5 min. The fat cells were harvested and incubated for various periods of time. After incubation the cells were separated from the medium by flotation in silicone (Prosil-28, PCR Research Chemicals, Inc., Gainesville, Fla.)-coated Pasteur pipettes and added to 0.5 ml of 0.4 N perchloric acid. An aliquot (500 μl) of the medium was deproteinized with the addition of 50 μl of 4.0 N perchloric acid. The perchlorate-treated cells and medium were centrifuged at 12,500 × *g* for 5 min and an aliquot of the supernatant was removed and neutralized with a slight molar excess of $KHCO_3$. Aliquots (100 μl) of the protein-free extracts were "streaked" onto cellulose MN 300 polyethyleneimine-impregnated plates (20 × 20 cm × 0.1 mm) (Brinkman CEL 300 PEI) and the plates were developed in *n*-butanol/acetic acid/water (2:1:1) (9). Standards of adenosine, inosine, hypoxanthine, and ATP were introduced onto the same chromatographic plates. Discrete areas of the plates associated with the standards were scraped from the plates and the nucleosides and nucleotides were extracted from the resin with 1 N HCl. Generally between 70% and 85% of the radioactivity introduced onto a chromatographic plate was recovered in the regions associated with the standards.

Partial purification of adenosine deaminase. Commercial preparations of adenosine deaminase were purified by gel chromatography on columns (20 × 1.4 cm) of Sephadex G-100. The columns were eluted with 10 mM sodium phosphate buffer (pH 7.4), and adenosine deaminase activity was determined by a decrease in absorbance at 265 nm. Purified adenosine deaminase was used in the experiments presented in Fig. 3 and Tables 3 and 5. For the remainder of the experiments, an unmodified commercial preparation was used.

RESULTS

The present studies were performed using clonidine because it has been shown to be the most effective *alpha*-adrenergic agonist on hamster adipose tissue (4). Table 1 presents data showing that lipolysis activated by 3-isobutyl-1-methylxanthine is inhibited by clonidine at concentrations as low as 10 nM, and is arrested at 1.0 μM. Similar responses to clonidine were observed at low and high densities of fat cells. In contrast, lipolysis activated by isoproterenol was significantly ($p < 0.05$) inhibited by clonidine only when the cell density was relatively low (<30,000 cells/ml). Additional data relating the effect of cell density to the antilipolytic activity of clonidine is presented in Fig. 1. The data contained in Fig. 1 are results from 26 experiments performed during

TABLE 1

Effect of cell density on antilipolytic action of clonidine

Hamster adipocytes were incubated at two different cell densities (>300,000 cells/ml or <30,000 cells/ml) for 60 min in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml. Isoproterenol, 3-isobutyl-l-methylxanthine (IBMX), and clonidine were present where indicated. Each value represents the mean ± standard error of at least four experiments.

	Glycerol production	
	>300,000 cells/ml	<30,000 cells/ml
	μmoles/g/30 min	
Basal	1.45 ± 0.41	4.71 ± 0.52
IBMX, 0.10 mM	9.45 ± 0.88	14.59 ± 1.21
+ Clonidine, 10 nM	6.74 ± 0.42[a]	5.91 ± 0.48[a]
+ Clonidine, 1000 nM	1.25 ± 0.15[a]	2.87 ± 0.32[a]
Isoproterenol, 1.0 μM	13.1 ± 1.21	12.7 ± 1.22
+ Clonidine, 10 μM	14.6 ± 1.55	9.9 ± 0.93[a]
+ Clonidine, 100 μM	14.8 ± 1.42	4.9 ± 0.54[a]

[a] Significantly different from value obtained with no added clonidine ($p < 0.05$ by paired Student's *t*-test).

a 6-month period. They show that the antilipolytic effect of clonidine is negatively correlated to the weight of cells present in each incubation vessel. The correlation coefficient of −0.56 was statistically significant ($p < 0.01$) using Student's *t*-test.

These observations suggested the existence of a substance that is elaborated from fat cells, accumulates in the incubation media, and influences the sensitivity of cells to clonidine. The experiment presented in Table 2 provides support for this possibility by showing that clonidine failed to inhibit isoproterenol-activated lipolysis when fat cells (<30,000 cells/ml) were incubated in media previously used for a cell incubation. The first incubation to prepare the "used" media lasted for 30 min and had a cell density of approximately 300,000 cells/ml. Freshly prepared adipocytes were used for the second incubation. In contrast to isoproterenol-activated lipolysis, 3-isobutyl-1-methylxanthine-accelerated lipolysis was inhibited by clonidine in both fresh and "used" media. This latter observation suggested to us the possibility that the endogenous antagonist of the action of clonidine is a purine derivative, since the *alpha*-agonist was effective in the presence of the purine analogue 3-isobutyl-1-methylxanthine.

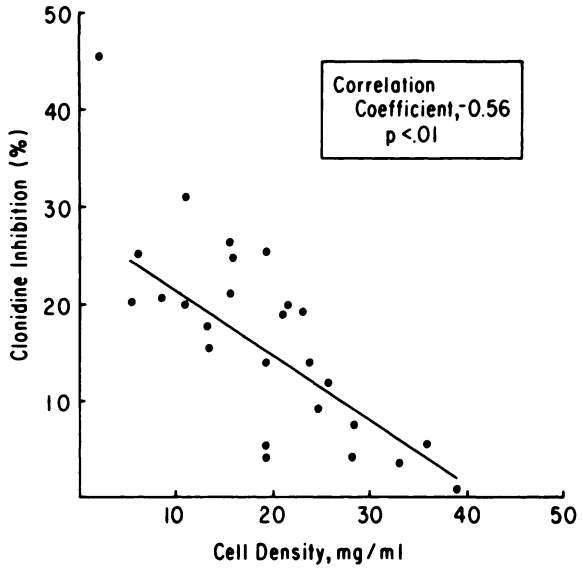

FIG. 1. *Effect of adipocyte density on antilipolytic activity of clonidine*

Adipocytes were incubated for 30 min in the presence of 1 μM isoproterenol with or without 10 μM clonidine. Cell density is expressed as milligrams of triglyceride per milliliter of medium. Basal lipolysis was 0.86 ± 0.07 μmole of glycerol per gram per 30 min; lipolysis in the presence of isoproterenol was 11.54 ± 0.97 μmoles/g/30 min, with a range of 7.25–21.73. The *line* was calculated by linear regression; the slope was −0.72.

Data provided by several laboratories (9–11) have shown that rat adipocytes release adenosine during incubation *in vitro*. Since methylxanthines have been shown to antagonize the action of adenosine on fat cells (12), experiments were performed to determine whether adenosine influenced the action of clonidine in hamster fat cells. These experiments were designed to compare the antilipolytic potency of clonidine with lipolysis activated by isoproterenol in the presence or absence of adenosine deaminase. Our rationale was that adenosine deaminase, by removing adenosine from incubation media, should enhance the antilipolytic effect of clonidine. However, because adenosine deaminase by itself can accelerate lipolysis and potentiate the lipolytic activity of isoproterenol (13), preliminary experiments were performed to determine the concentrations of adenosine deaminase that would elicit a submaximal stimulation of both lipolysis and accumulation of cyclic AMP in the presence of isoproterenol. Figure 2 presents data showing the effects of various concentrations of adenosine deaminase on the stimulation of lipolysis and the accumulation of cyclic AMP in adipocytes exposed to isoproterenol. None of the adenosine deaminase concentrations used stimulated lipolysis or accumulation of cyclic AMP. However, the lipolytic and cyclic AMP responses to isopro-

TABLE 2

Inhibition of lipolysis by clonidine: comparison of fresh and used media

Hamster adipocytes (approximately 30,000 cells/ml) were incubated for 60 min in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml. Isoproterenol, 3-isobutyl-l-methylxanthine (IBMX), and clonidine were present where indicated. Media designated "fresh" were prepared immediately prior to use; media designated "used" were recovered from a previous cell incubation lasting for 30 min and having a cell density of greater than 300,000 cells/ml. Each value represents the mean ± standard error of three experiments.

	Glycerol production	
	Control	Clonidine, 10 μM
	μmoles/g/30 min	
Fresh Media		
Basal	3.21 ± 0.24	1.31 ± 0.22
Isoproterenol, 1.0 μM	7.6 ± 0.41	3.6 ± 0.32[a]
IBMX, 0.10 mM	12.2 ± 0.99	4.6 ± 0.58[a]
Used Media		
Basal	0.29 ± 0.06	0.18 ± 0.04
Isoproterenol, 1.0 μM	5.0 ± 0.68	4.8 ± 0.52
IBMX, 0.10 mM	9.0 ± 1.03	4.4 ± 0.60[a]

[a] Significantly different from value obtained with no added clonidine ($p < 0.05$ by paired Student's *t*-test).

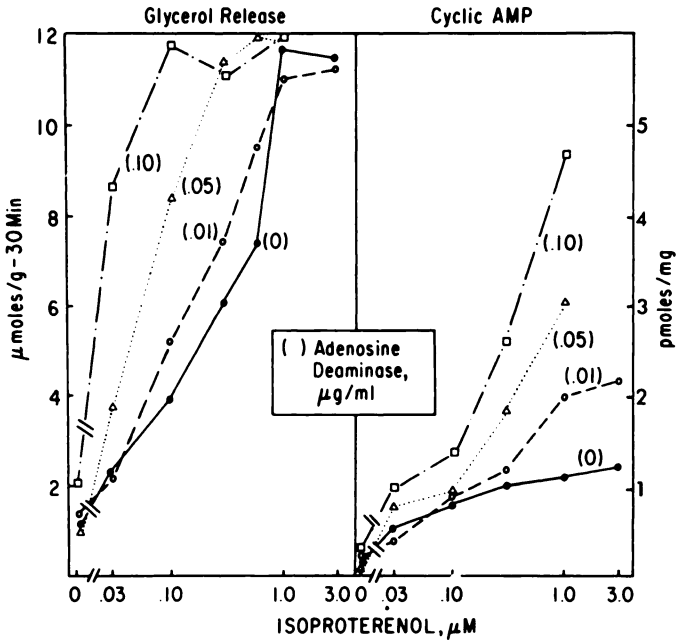

FIG. 2. *Effect of adenosine deaminase on lipolysis and accumulation of cyclic AMP in response to isoproterenol*

Adipocytes (19–28 mg) were incubated for 30 min in the absence or presence of purified adenosine deaminase and various concentrations of isoproterenol. The concentration of adenosine deaminase is indicated in parentheses adjacent to each line. Each value represents the average of three separate experiments.

terenol were potentiated by adenosine deaminase in a dose-dependent fashion. Adenosine deaminase increased the maximal cyclic AMP response but not the maximal lipolytic response to isoproterenol. These results were used in the design of subsequent experiments by indicating the concentrations of isoproterenol needed to produce equivalent degrees of activation of lipolysis and accumulation of cyclic AMP in the absence or presence of adenosine deaminase.

Figure 3 presents data showing the effect of clonidine on lipolysis and cyclic AMP levels in cells incubated with either isoproterenol alone or with isoproterenol and adenosine deaminase. In the absence of enzyme, clonidine did not antagonize isoproterenol-activated lipolysis but, in the presence of adenosine deaminase, lipolysis was dramatically inhibited by clonidine. However, the antilipolytic effect of clonidine was overcome by increasing the concentration of isoproterenol. Cyclic AMP accumulation was increased to a greater extent in the presence of adenosine deaminase. Clonidine did not affect cyclic AMP levels when isoproterenol was present alone, but the *alpha*-adrenergic agonist lowered cyclic AMP accumulation when adenosine deaminase was added. In this study, adenosine deaminase (0.10 μg/ml) by itself did not activate lipolysis (control, 1.34 ± 0.25; adenosine deaminase, 2.01 ± 0.47 μmoles of glycerol per gram per 30 min).

Table 3 presents the relationship between the concentration of adenosine deaminase and the antilipolytic effect of clonidine. In the absence of adenosine deaminase, clonidine inhibited lipolysis activated by isoproterenol by less than 20%. The addition of adenosine deaminase, 0.001 μg/ml, doubled the antilipolytic activity of clonidine and, in the presence of an enzyme concentration of 0.01 μg/ml, clonidine inhibited lipolysis by nearly 70%. In this experiment, nearly equivalent rates of lipolysis were obtained at each concentration of adenosine deaminase by decreasing the concentration of isoproterenol as the concentration of enzyme was increased. The rate of lipolysis obtained represents approximately 75% of the maximal rate observed with the combination of 1.0 μM isoproterenol and adenosine deaminase, 1.0 μg/ml. Cyclic AMP levels increased slightly as the concentration of adenosine deaminase was increased (Table 3). Clonidine did not lower cyclic AMP levels increased by isoproterenol alone, but became progressively more effective at retarding accumulation of the cyclic nucleotide as the concentration of adenosine deaminase was increased.

The data presented in Fig. 4 indicate that clonidine inhibition of lipolysis and accumulation of cyclic AMP was also enhanced in the presence of theophylline. As was seen when adenosine deaminase was used, the antilipolytic effect of clonidine was overcome at high isoproterenol concentrations. In this experiment, a concentration of theophylline was used (0.050 mM) which did not by itself increase lipolysis (control, 0.48 ± 0.07 μmoles/g/30 min; theophylline, 0.73 ± 0.19 μmoles/g/30 min).

The experiment presented in Table 4 studied the effect of adenosine deaminase on the antilipolytic activity of other *alpha*-adrenergic agonists. The activity of methoxamine is similarly enhanced in the presence of adenosine deaminase. In contrast, phenylephrine appeared to inhibit lipolysis to an equivalent extent in the presence or absence of adenosine deaminase.

Our next experiment examined the antilipolytic effects

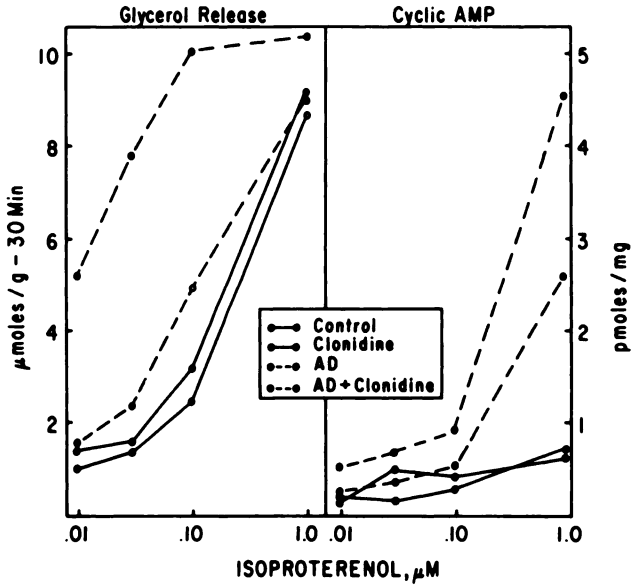

FIG. 3. *Effect of clonidine on lipolysis and accumulation of cyclic AMP in the presence or absence of adenosine deaminase*

Adipocytes (19–31 mg) were incubated for 30 min in the absence or presence of unmodified adenosine deaminase (0.10 μg/ml). Clonidine was present where indicated at a concentration of 10 μM. Basal lipolysis in the absence of adenosine deaminase was 1.34 ± 0.25, and 2.01 ± 0.47 μmole of glycerol per gram per 30 min in the presence of the enzyme. Cyclic AMP levels were as follows: no enzyme, 0.23 ± 0.3; with enzyme, 0.29 ± 0.03 pmole/mg. Each value represents the average of three separate experiments.

TABLE 3

Effect of adenosine deaminase on antilipolytic and cyclic AMP-suppressive activity of clonidine

Hamster adipocytes (14–21 mg) were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml. Adenosine deaminase and isoproterenol were present at the indicated concentrations. Clonidine was present at a concentration of 10 μM. Each value represents the mean of three experiments, performed on separate days.

Additions		Glycerol production			Cyclic AMP		
Adenosine deaminase	Isoproterenol	Control	Clonidine	Δ	Control	Clonidine	Δ
μg/ml	*μM*	*μmoles/g/30 min*			*pmoles/mg*		
0	0	0.82	0.91		0.21	0.20	−0.01 ± 0.03
0	0.60	11.73	9.85	−1.99 ± 0.77	1.05	1.09	0.04 ± 0.06
0.001	0.60	14.21	8.83	−5.38 ± 1.18[a]	1.07	1.12	0.05 ± 0.07
0.005	0.60	15.91	7.96	−7.96 ± 1.35[a]	1.15	1.00	−0.15 ± 0.08
0.010	0.30	10.68	4.06	−6.62 ± 1.11[a]	1.47	1.02	−0.45 ± 0.10[a]
0.050	0.30	11.21	4.25	−6.95 ± 1.31[a]	1.47	0.71	−0.76 ± 0.16[a]
0.100	0.10	9.93	4.01	−5.92 ± 0.77[a]	1.37	0.75	−0.62 ± 0.13[a]
0.500	0.10	11.75	3.76	−7.99 ± 0.81[a]	1.41	0.73	−0.68 ± 0.13[a]
1.00	0.10	13.44	4.03	−9.41 ± 0.86[a]	1.70	0.68	−1.02 ± 0.11[a]

[a] Significantly different from value obtained with no added clonidine ($p < 0.05$ by paired Student's *t*-test).

of nicotinic acid and prostaglandin E_1. As was the case when clonidine was used, lipolysis activated by 3-isobutyl-1-methylxanthine is more sensitive to inhibition by either nicotinic acid or prostaglandin E_1 than is isoproterenol-activated lipolysis (Fig. 5). Cyclic AMP accumulations in response to isoproterenol or 3-isobutyl-1-methylxanthine were similar (isoproterenol, 0.92 ± 0.11 pmole/mg; 3-isobutyl-1-methylxanthine, 0.81 ± 0.13 pmole/mg). Moreover, the antilipolytic activity of both nicotinic acid and prostaglandin E_1 against isoproterenol

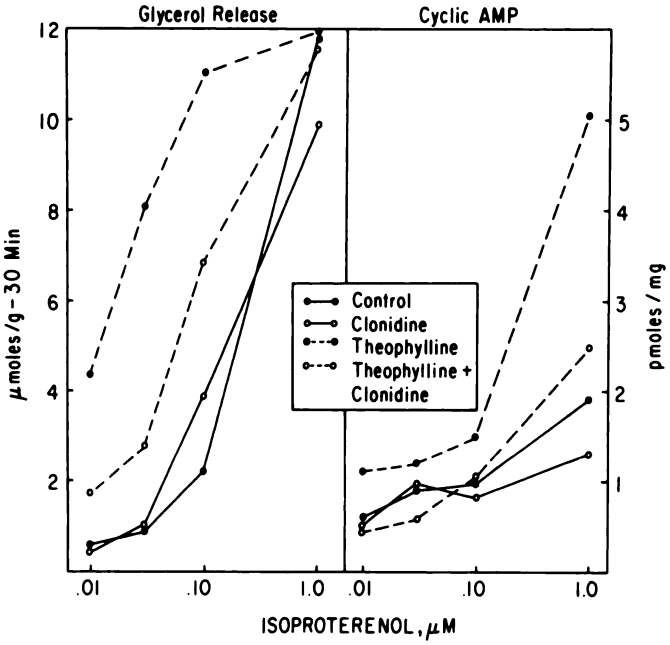

FIG. 4. *Effect of clonidine on lipolysis and accumulation of cyclic AMP in the presence or absence of theophylline*

Adipocytes (17–26 mg) were incubated for 30 min in the absence or presence of theophylline, 0.050 mM. Clonidine was present where indicated at a concentration of 10 μM. Basal lipolysis in the absence of theophylline was 0.48 ± 0.07 μmole/g/30 min, and 0.73 ± 0.10 μmole/g/30 min with theophylline. Cyclic AMP levels were as follows: no theophylline, 0.24 ± 0.03 pmole/mg; with theophylline, 0.39 ± 0.05 pmole/mg. Each value represents the average of four separate experiments.

activated lipolysis is increased in the presence of adenosine deaminase (Table 5). Cyclic AMP levels were also suppressed to a greater extent in cells exposed to adenosine deaminase. In contrast, inhibition of lipolysis by insulin was unaffected by the addition of adenosine deaminase.

Our interpretation of the foregoing experiments assumes that adenosine accumulates in media in which hamster fat cells are incubated. The results presented in Table 6 demonstrate the validity of this assumption. In this study, the nucleotide pool of fat cells was labeled with either [^{14}C]- or [^{3}H]adenine prior to digestion with collagenase. During the following incubation, the adipocytes released radioactive adenosine into the incubation media. Adenosine accumulation was maximal at 5 min and 30 min; after 60 min adenosine was still present in the incubation media. The addition of adenosine deaminase at concentrations that enhanced clonidine activity (i.e., 1.0 ng/ml or 1.0 μg/ml) greatly reduced the accumulation of radiolabeled adenosine in incubation media. Adenosine was still detected in incubation media that was permitted to remain at room temperature for 15 or 60 min prior to deproteinization with perchlorate (control, 51.6 cpm adenosine/mg; 15 min, 49.1 cpm/mg; 60 min, 57.4 cpm/mg).

DISCUSSION

In the present study, we report that clonidine, prostaglandin E_1, and nicotinic acid are not strongly antilipolytic when lipolysis is accelerated by isoproterenol alone. In contrast, when lipolysis is increased by a lower concentration of isoproterenol in combination with either adenosine deaminase or theophylline, clonidine, prostaglandin E_1, and nicotinic acid are markedly antilipolytic. Although it is generally agreed that cyclic AMP levels must be rate-limiting for lipolysis in order to detect inhibitory effects of clonidine, prostaglandin E_1, or nicotinic acid (14), establishing the rate-limiting concentrations of cyclic AMP in a particular experiment is difficult. In the present study, the levels of cyclic AMP are almost identical in the two experimental conditions, as are the

TABLE 4

Inhibition of lipolysis by selective alpha-adrenergic agonists in the presence or absence of adenosine deaminase

Hamster adipocytes (18–31 mg/ml) were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml. Basal lipolysis was measured to be 1.22 ± 0.09 μmoles/g/30 min. In the absence of adenosine deaminase, lipolysis was activated by 0.6 μM isoproterenol; in the presence of adenosine deaminase, 0.10 μg/ml, by 0.10 μM isoproterenol. Clonidine and methoxamine were present at concentrations of 30 μM, phenylephrine at a concentration of 100 μM. Each value represents the mean ± standard error of three experiments, performed on separate days.

Alpha-agonist	Glycerol production			
	Minus adenosine deaminase	Δ	Plus adenosine deaminase	Δ
	μmoles/g/30 min			
None	13.35 ± 1.25		12.02 ± 1.33	
Clonidine	11.38 ± 1.73	−1.97 ± 0.51	7.21 ± 0.68	−4.81 ± 0.57[a]
Methoxamine	10.60 ± 1.16	−2.75 ± 0.59[a]	7.53 ± 0.72	−4.48 ± 0.61[a]
Phenylephrine	8.49 ± 0.78	−4.85 ± 0.47[a]	8.02 ± 0.91	−3.99 ± 0.41[a]

[a] Significantly different from zero ($p < 0.05$ by paired Student's *t*-test).

rates of lipolysis, which were lower than maximal. These data seem to exclude the possibility that the failure of clonidine, prostaglandin E_1, or nicotinic acid to inhibit lipolysis in the absence of adenosine deaminase results from excessively high cyclic AMP levels (14). Indeed, inspection of the data reveals that cyclic AMP levels were slightly higher when adenosine deaminase was present (Figs. 2 and 3; Table 3).

We (4) and others (15–18) have reported that methylxanthine-activated lipolysis is more sensitive to inhibition by clonidine, prostaglandin E_1, and nicotinic acid then is catecholamine-activated lipolysis. Studies from several different laboratories have shown that one mechanism of methylxanthine action involves blockade of specific adenosine receptors (12, 19). Fat cells produce adenosine (9–11), which inhibits adenylate cyclase and lipolysis (13)—effects that are prevented by methylxanthines (12, 13). Since catecholamine-activated lipolysis is only marginally inhibited by exogenous adenosine (20, 21), we suggest that sufficient adenosine is formed to activate fully the antilipolytic mechanism regulated by the purine nucleoside. Catecholamine-activated lipolysis is also only slightly inhibited by clonidine, prostaglandin E_1, and nicotinic acid, suggesting, therefore, that the antilipolytic mechanism regulated by adenosine is also regulated by these agents. In extending this line of reasoning, the sensitivity of lipolysis to inhibition would be enhanced by retarding adenosine accumulation, such as by diluting the cells, preventing adenosine accumulation with adenosine deaminase, or blocking adenosine action, such as with methylxanthines (12). Our results show increased sensitivity of fat cells to clonidine under each of these conditions. Similar data were presented by Fain *et al.* (22) and Wieser and Fain (23), who showed over 90% inhibition of lipolysis activated by norepinephrine (0.10 μM) and adenosine deaminase (1 μg/ml) with N^6-(phenylisopropyl)adenosine, whereas lipolysis activated to a similar extent by norepinephrine (1.5 and 3.3 μM) alone was inhibited with N^6-(phenylisopropyl)adenosine to only 50%.

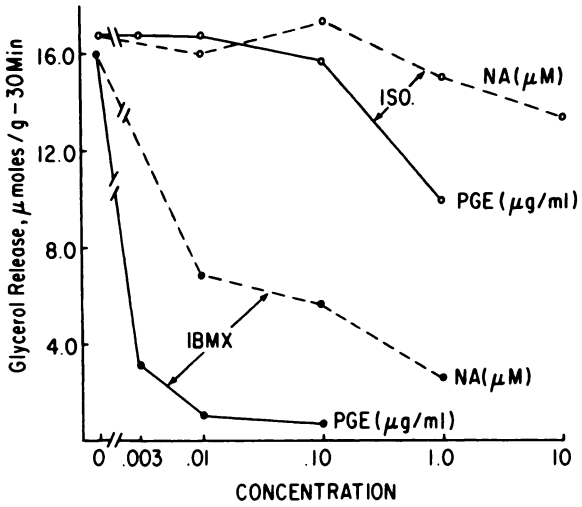

FIG. 5. *Effect of prostaglandin E_1 and nicotinic acid on lipolysis in hamster adipocytes*

Hamster adipocytes (21–25 mg) were incubated for 30 min with either 1.0 μM isoproterenol or 0.10 mM 3-isobutyl-1-methylxanthine (*IBMX*). Basal lipolysis was 1.03 μmoles/g/30 min. Each value represents the mean of three experiments.

Although our results do not determine the molecular mechanisms underlying the interaction among adenosine, clonidine, nicotinic acid, and prostaglandin E_1, it is likely that inhibition of adenylate cyclase is involved. Hormone regulation of adenylate cyclase is mediated by regulatory proteins that bind GTP (24). Two nucleotide regulatory proteins have been proposed: one mediating stimulatory effects on adenylate cyclase, the other mediating inhibitory effects (24). On the assumption that the inhibitory effects of clonidine, adenosine, prostaglandin E_1, and nicotinic acid on adipocyte adenylate cyclase are exerted through a common regulatory protein, we suggest that fat cells may be unresponsive to clonidine, nicotinic acid, and prostaglandin E_1 because the regulatory protein that mediates inhibition of adenylate cyclase is held in an active state by endogenous adenosine and cannot be activated further. In support of this contention, Cooper and Londos (25) showed that the sensitivity of adipocyte adenylate cyclase to inhibition by N^6-(phenylisopropyl) adenosine was increased by inclusion of adenosine deaminase in their assay. In addition, Aktories *et al.* (6) reported inhibition of hamster adipocyte adenylate cyclase with *alpha* agents, prostaglandin E_1, and nicotinic acid under conditions that prevented adenosine action by inclusion of 3-isobutyl-1-methylxanthine in the assay.

TABLE 5

Inhibition of lipolysis and cyclic AMP accumulation by clonidine, prostaglandin E_1, nicotinic acid, and insulin in the presence or absence of adenosine deaminase

Hamster adipocytes (18–29 mg/ml) were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing bovine serum albumin, 40 mg/ml. The adenosine deaminase was purified by chromatography on Sephadex G-100. In the absence of adenosine deaminase, lipolysis was activated by 0.60 μM isoproterenol; in the presence of adenosine deaminase (0.10 μg/ml) by 0.30 μM isoproterenol. Each value represents the mean ± standard error of four experiments performed on separate days.

	Glycerol release		Cyclic AMP	
	Minus adenosine deaminase	Plus adenosine deaminase	Minus adenosine deaminase	Plus adenosine deaminase
	μmoles/g/30 min		*pmoles/mg*	
Basal	1.41 ± 0.17	1.73 ± 0.25	0.15 ± 0.01	0.16 ± 0.01
Isoproterenol	14.58 ± 1.54	15.61 ± 1.65	1.6 ± 0.24	1.9 ± 0.31
Δ Due to clonidine, 10 μM	−1.22 ± 0.37	−5.48 ± 0.67[a]	−0.20 ± 0.25	−0.90 ± 0.21[a]
Δ Due to prostaglandin E_1, 0.10 μg/ml	−2.68 ± 0.64	−8.49 ± 0.71[a]	+0.50 ± 0.40	−1.10 ± 0.25[a]
Δ Due to nicotinic acid, 1.0 μM	−3.07 ± 0.73	−6.48 ± 0.83[a]	−0.20 ± 0.35	−1.0 ± 0.23[a]
Δ Due to insulin, 100 μU/ml	−3.41 ± 0.44[a]	−3.65 ± 0.31[a]	−0.50 ± 0.14[a]	−0.90 ± 0.18[a]

[a] Significantly different from zero ($p < 0.05$ by paired Student's *t*-test).

The validity of the preceding discussion assumes an important role of the adenylate cyclase-cyclic AMP system in *alpha*-adrenergic inhibition of lipolysis. A number of observations are consistent with cyclic AMP mediation of *alpha*-adrenergic inhibition of lipolysis. Suppression of cyclic AMP levels generally accompanies *alpha*-adrenergic inhibition of lipolysis (Figs. 3 and 4; Tables 3–5). Conversely, conditions that prevent clonidine inhibition of lipolysis also prevent clonidine suppression of cyclic AMP levels (Figs. 3 and 4; Table 3). However, suppression of cyclic AMP does not always accompany *alpha*-adrenergic inhibition of lipolysis, as is shown in Table 3 at adenosine deaminase concentrations of 0.001 μg/ml and 0.005 μg/ml. This apparent dissociation between cyclic AMP levels and lipolysis is reminiscent of the inability of many laboratories to detect suppression of cyclic AMP accompanying insulin inhibition of lipolysis (26, 27). It is quite possible that insulin, *alpha*-adrenergic agonists, and other antilipolytic compounds affect lipolysis by mechanisms in addition to and independent of cyclic AMP (2, 22, 28). Nevertheless, it seems certain that one of the effects of *alpha*-adrenergic agents is to lower cyclic AMP levels (2–6) and that this is sufficient to account in part for their antilipolytic activity.

The minimum effective concentration of adenosine deaminase required to enhance clonidine activity was 1.0 ng/ml (Table 3), a concentration calculated to deaminate approximately 0.24 nmole of adenosine per minute. Unfortunately, there are no reliable estimates of the rate of adenosine production from fat cells to compare with this enzyme activity. Two reports observed adenosine release from fat cells in appreciable amounts (10, 11), but Fain (9) could detect release of adenosine only by labeling the adenine nucleotide pool of the cells, a procedure similar to that employed in the present experiments. Fain (9) has argued that failure to detect adenosine in media of a fat cell incubation is caused by adenosine deaminase contamination of commercial albumin preparations. The results presented in Table 6 showing accumulation of adenosine in incubation media and its removal by added adenosine deaminase suggest that the albumin used for the present studies was not contaminated with adenosine deaminase. This view is supported by the finding that adenosine was still detected in media that remained at room temperature for 1 h prior to deproteinization. This observation is important because the "used" media for the experiments presented in Table 2 were not reused immediately upon completion of the first incubation. All of our experiments used the same preparation of bovine serum albumin (Sigma, Lot 97C-0411), and hence we are unable to comment upon the possibility that other preparations of albumin from this or other suppliers may have been contaminated with adenosine deaminase.

In the present study, insulin inhibition of lipolysis was not improved by addition of adenosine deaminase (Table 5). Schwabe *et al.* (29) demonstrated that insulin failed to antagonize norepinephrine-activated lipolysis in dilute adipocyte suspensions and that responsiveness to insulin

TABLE 6

Production of radiolabeled adenosine from hamster adipocytes

Hamster adipose tissue was labeled in the presence of [^{14}C]- or [^{3}H]adenine prior to digestion with collagenase. The isolated fat cells were washed, and 40–50 mg of cells were incubated in a volume of 1.0 ml for the times indicated. Purified adenosine deaminase was present in the concentration indicated. The data are expressed as counts per minute of adenosine present in each milliliter of incubation medium per milligram of cells present. Each value represents the mean ± error standard of the number of experiments indicated in parentheses.

	Adenosine production		
	5 Min	30 Min	60 Min
		cpm/mg	
Control	37.8 ± 7.7 (4)	41.3 ± 6.08 (5)	17.0 ± 3.16 (5)
Adenosine deaminase, 1.0 μg/ml		2.17 ± 1.8 (4)	
Adenosine deaminase, 1.0 ng/ml		6.49 ± 3.9 (4)	

was restored by adenosine. Green and Newsholme (30) reported that lipolysis accelerated by adenosine deaminase was less sensitive to inhibition with insulin than was norepinephrine-activated lipolysis. Taken together, these observations suggest that insulin inhibition of lipolysis is facilitated by adenosine. Interestingly, the antilipolytic activity of phenylephrine similarly is not improved by adenosine deaminase (Table 5), suggesting that the mechanism of antilipolytic action of this agent differs from that of the other *alpha* agonists used. A similar proposal was advanced in a previous publication (4) which documented a lack of interaction in the antilipolytic effect of clonidine and phenylephrine when present simultaneously in fat cell incubations. However, yohimbine more effectively blocked clonidine and phenylephrine inhibition of lipolysis than did prazosin, suggesting that both *alpha*-agonists influence lipolysis through *alpha*$_2$ receptors (4).

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Send reprint requests to: Dr. Richard J. Schimmel, Department of Physiology, College of Medicine and Dentistry of New Jersey, New Jersey School of Osteopathic Medicine, Piscataway, N. J. 08854.