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Effects of Epinephrine, Adrenocorticotrophic Hormone, and Theophylline on Adenosine 3′,5′-Monophosphate Phosphodiesterase Activity in Fat Cells

(insulin/cyclic AMP)

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ABSTRACT **When rat fat cells were incubated with ACTH, epinephrine, or theophylline for 2 to 10 min, cyclic AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, E.C. 3.1.4.17) activity (K_m about 0.39 μM) in the 100,000 × *g* sediment fraction of homogenates was increased 35 to 50%. The effects of epinephrine and ACTH were concentration dependent and maximal increases were produced with concentrations similar to those that maximally stimulate lipolysis. Theophylline (0.5 mM) similarly increased phosphodiesterase activity but did not enhance the effects of maximally effective concentrations of the hormones. The changes in phosphodiesterase activity following addition of ACTH or theophylline paralleled changes in cell cyclic AMP content; both reached a maximum within 5 min and then declined, approaching basal levels after 20 or 30 min. The increased phosphodiesterase activity in cells incubated for 5 min with epinephrine reverted to basal levels within 2.5 min after the addition of propranolol. Our data are consistent with the view that there is a component of the fat-cell phosphodiesterase, perhaps localized in the plasma membrane, whose activity can be acutely modified by the concentration of its substrate, cyclic AMP.**

As previously reported (*J. Biol. Chem.* 248, 7164–7170, 1973), exposure of fat cells to insulin increases the activity of a low K_m phosphodiesterase also localized in the 100,000 × *g* sediment fraction of fat cell homogenates. In the presence of insulin, however, phosphodiesterase remained elevated for at least 40 min and there was no significant change in fat-cell cyclic AMP content. When phosphodiesterase activity was elevated and cyclic AMP content maintained at a high level by incubation of cells with ACTH plus theophylline, insulin produced a further increase in enzyme activity. Whether or not insulin and ACTH (or epinephrine or theophylline) affect the same phosphodiesterase, there seems little doubt that the underlying mechanisms are different.

When rat fat cells are incubated *in vitro*, the addition of epinephrine (or ACTH) in suitable concentration produces a rapid rise in intracellular cyclic AMP concentration which reaches a maximum in 4 to 5 min and declines thereafter (1, 2). Earlier studies in this laboratory (2) established that hormonal stimulation of adenylate cyclase continued during the period that cyclic AMP concentration was decreasing, but attempts to demonstrate an increase in phosphodiesterase activity that might account for the rapid reversal of the initial accumulation of cyclic AMP were unsuccessful (2). We have now found, as reported below, that the activity of a particulate phosphodiesterase with a high affinity for cyclic AMP can be rapidly and reversibly increased by exposure of fat cells to epinephrine, ACTH or theophylline.† It appears, at least in the case of this specific phosphodiesterase, that cyclic AMP, in addition to serving as a substrate, may play a role in the acute regulation of catalytic activity.

METHODS

Epididymal fat pads were obtained from Osborne-Mendel rats (125–150 g) that were permitted free access to food and water until they were decapitated. Krebs-Ringer phosphate medium containing 5 mM glucose and 30 mg/ml albumin (fraction *V* from bovine serum) was used for the preparation (3), washing, and incubation of cells, all of which were carried out at 37°. Samples (2 or 3 ml) of the suspension of washed fat cells (containing 50 to 75 mg of cells per ml) were distributed to polyethylene vials and incubated for 15 to 30 min. Additions of hormones or other agents were then made, and the incubation period was terminated at the indicated time thereafter by forcing the cells (plus medium) 6 times through a 13 steel mesh screen using a Swinny adapter (XY, 3001200) and a polyethylene syringe. The incubation vial was washed with 3 ml 50 mM Tris·HCl buffer (pH 8.0), which was then used to wash the mesh and syringe, and was finally added to the homogenate. After centrifugation at 100,000 × *g* for 30 min (4°), the floating fat was removed with a spatula, the supernatant decanted, and the pellet dispersed in 50 mM Tris·HCl buffer (pH 8.0) using either a Dounce homogenizer or a Polytron (Brinkman Instruments). Phosphodiesterase activity in the latter fraction (100,000 × *g* pellet) was assayed in triplicate (4) with 0.55 μM cyclic AMP as substrate, unless otherwise noted. In some experiments, 5′-nucleotidase activity was also assayed (4) with 0.55 μM and 55 μM 5′-AMP as substrate. Enzyme activities are reported in μ units equal to 1 pmole of substrate hydrolyzed per minute. Protein was determined by the method of Lowry et al. (5) using bovine serum albumin as a standard.

For experiments in which cyclic AMP was measured, several samples of fat cells were incubated under each set of conditions. At the indicated times, two samples were prepared for assay of phosphodiesterase as described above and 0.8 ml of cells plus medium were transferred from each of two other incubations to glass tubes containing 0.1 ml of 50% trichloroacetic (w/v) and [^{3}H]cyclic AMP (about 4,000 cpm). After

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† An apparently similar effect of epinephrine on fat-cell phosphodiesterase has been reported in abstract form [Zinman, B. & Hollenberg, C. H. (1972) *Excerpta Medica International Congress Series* **256**, 157 Abstr.].

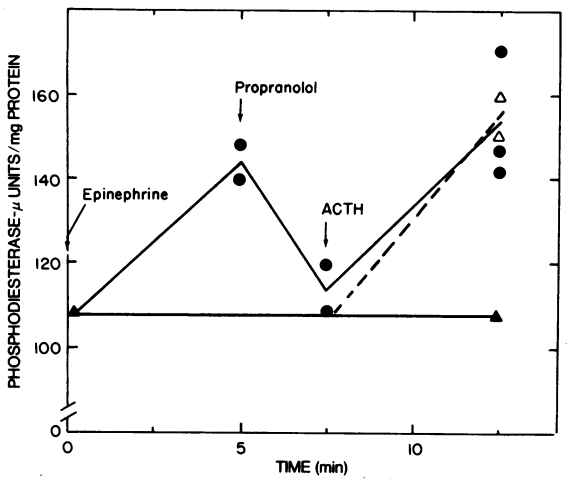

FIG. 1. Effect of epinephrine, propranolol, and ACTH on phosphodiesterase activity. After incubation for 5 min with (●) or without (▲) 0.5 μM epinephrine, 8 μM propranolol was added to the vials containing epinephrine. ACTH (250 mU/ml) was added 2.5 min later to those vials (●) and to cells not previously exposed to epinephrine and propranolol (△).

stirring, 0.5 ml of 5% trichloroacetic acid was added and the tubes centrifuged. The sedimented material was washed with 0.5 ml of 5% trichloroacetic acid and, after centrifugation, the supernatant was added to the first supernatant. After addition of 0.02 ml of 10 N HCl, each extract was transferred to a column (0.5 × 6.5 cm) of AG 50WX8 (H^+ form, 100–200 mesh) previously washed with 5 ml of 0.5 N HCl, 10 ml of water, and 5 ml of 0.1 N HCl. After the addition of 5.5 ml of 0.1 N HCl and 1 ml of water (eluates discarded), cyclic AMP was eluted with 8 ml of water. This eluate was applied to a column (0.7 × 2.5 cm) of AG 1X2 (Cl^- form, 100–200 mesh), followed by 5 ml of water and 1 ml of 0.1 N HCl (eluates discarded). Then, 2 ml of 0.1 N HCl was added and the eluate collected and lyophylized. Samples of the residue dissolved in 150 μl of water were used for assay of cyclic AMP by the method of Gilman (6) and for radioassay of ^3H to assess recovery. The fat-cell cyclic AMP levels observed in the studies reported here are considerably lower than those found in similar experiments

TABLE 1. *Subcellular distribution of phosphodiesterase activity*

Fractions of homogenate after centrifugation	Phosphodiesterase activity (μunits/mg of protein)		
	Control	ACTH	Epinephrine
5,000 × *g* sediment	46	45	39
supernatant	94	106	103
100,000 × *g* sediment	151	196	186
supernatant	53	54	54

Cells were incubated for 10 min after addition of 250 mU/ml ACTH, or 2.7 μM epinephrine, then washed 3 times with 0.3 M sucrose–50 mM Tris·HCl (pH 8.0), with or without hormone, and homogenized in the same media. The homogenates were centrifuged at 5000 × *g* for 5 min, the fat layers removed, and the supernatants decanted. A sample of the supernatant was then centrifuged at 100,000 *g* for 30 min. The pellet fractions were suspended in the sucrose–Tris solution. Samples of each fraction were used for measurement of protein content and phosphodiesterase activity (with 0.5 μM cyclic AMP).

TABLE 2. *Effect of ACTH on Michaelis constants of phosphodiesterase from fat cells*

Additions	K_m-μM	V_{max}-μunits/mg of protein
None	0.39 ± 0.07	173.8 ± 25.2
ACTH (50 mU/ml)	0.45 ± 0.07	265.0 ± 31.9
p*	<0.01	<0.01

Fat cells were incubated for 10 min with or without ACTH. The 100,000 × *g* sediment fractions were suspended in a solution of 10 mM Tris·HCl (pH 8.0) in 180 mM sucrose. Preparations from duplicate incubations with or without ACTH were pooled and samples were used for assay of phosphodiesterase activity with cyclic AMP concentrations from 0.05 to 0.5 μM and from 40 to 200 μM. Incubation time and amount of enzyme were varied to permit estimation of initial rates. Kinetic parameters were derived from regression lines of S/v against S, calculated by the "least squares" method. In most experiments, relatively little activity was observed at high substrate concentrations, other than that attributable to the low K_m (0.39) component of the enzyme. As calculated after subtraction of V_{max} for the low K_m activity from velocities observed at high substrate concentrations (7), the higher K_m (10 to 50 μM) and V_{max} were not significantly altered by ACTH. Data presented are the SEM of values from 3 experiments.

* Based on paired differences between control and ACTH treated.

several years ago. The reasons for this difference are unclear but appear not be related to any differences in the methods used for extraction and purification of cyclic AMP; all earlier findings have been qualitatively reproduced.

All observations reported here have been replicated at least twice with different preparations of fat cells, but in several instances only data from individual representative experiments are presented. Hormones and other reagents were obtained as described previously (4).

RESULTS

As shown in Table 1, exposure of fat cells to epinephrine or ACTH for 10 min produced no significant change in phosphodiesterase activity assayed in pellet and supernatant fractions separated from homogenates by centrifugation at 5000 × *g* for 5 min. Activity in the final supernatant obtained after centrifugation for 30 min at 100,000 × *g* was likewise unchanged, while in sediment fractions from cells incubated with epinephrine or ACTH the specific activity of phosphodiesterase was significantly greater than it was in the 100,000 × *g* pellet from control cells. As the total activity of the first pellet fraction was only about 20% of that in the second, a single centrifugation (100,000 × *g* for 30 min) was used to obtain the particulate fractions for assay in the other experiments reported here. Assays (with 0.5 μM cyclic AMP) of sediment fractions from control and ACTH-treated cells plus supernatant fractions from both types of cells yielded the activities to be expected, based on addition of the activities of the individual fractions. Incubation of fat cells with ACTH (or epinephrine) did not alter the recovery of protein in the 100,000 × *g* sediment or the specific activities of 5′-nucleotidase and adenylate cyclase in this fraction (data not shown).

Phosphodiesterase activity in the particulate fraction usually exhibited two Michaelis constants for cyclic AMP but

TABLE 3. *Effects of hormones and theophylline on phosphodiesterase activity*

Experiment Number	Addition (concentration)	Theophylline (mM)	Phosphodiesterase (μunits/mg of protein)
1	None	0	109 (± 0)
	ACTH (0.5 mU/ml)	0	120
	(1.0 mU/ml)	0	131
	(5.0 mU/ml)	0	142
	(10 mU/ml)	0	143
	(50 mU/ml)	0	138
2	None	0	117 (± 0)
		0.5	142
	Epinephrine (2.7 μM)	0	145
		0.5	135
	ACTH (250 mU/ml)	0	143 (± 1)
		0.5	138
	Epinephrine (2.7 μM) plus ACTH (250 mU/ml)	0	148 (± 0)
		0.5	143 (± 1)
3	None	0	64 (± 5)
		0.5	97 (± 1)
		1.0	103 (± 8)
	ACTH (250 mU/ml)	0	104 (± 2)
		0.5	102 (± 4)
		1.0	94 (± 1)
4	None	0	52 (± 4)
		0.5	74 (± 2)
	ACTH (250 mU/ml)	0	89 (± 1)
		0.5	88 (± 1)
	Insulin (1 mU/ml)	0	78 (± 4)
	ACTH (250 mU/ml) plus insulin (1 mU/ml)	0.5	102 (± 4)
5	None	0	114 (± 4)
		0.5	161 (± 1)
	ACTH (250 mU/ml)	0	160 (± 20)
		0.5	164
	Insulin (1 mU/ml)	0	162
	ACTH (250 mU/ml) plus insulin (1 mU/ml)	0.5	225 (± 9)

Cells were incubated for 30 min before additions were made. (Theophylline, when present, was introduced immediately before other additions.) Incubation was then continued for 7.5 min and particulate fractions were prepared as described in *Methods*. Phosphodiesterase activity is reported as the value from a single sample of cells or the mean of values from duplicate samples ± one-half the range in parentheses.

V_{max} for the high K_m (20–50 μM) activity was never more than twice that for the low K_m and in some instances essentially no higher K_m activity was apparent. As shown in Table 2, ACTH caused a small but statistically significant increase in the low K_m, and a marked increase in V_{max} of the high affinity phosphodiesterase. No effects on the higher K_m activity were demonstrable.

The magnitude of the increment in phosphodiesterase activity after incubation of cells for 7.5 min with ACTH was dependent on hormone concentration (Table 3, Experiment 1) over a range similar to that in which we have found lipolysis to be a direct function of the log of hormone concentration.‡ This was true also for epinephrine, which produced maximal

‡ Manganiello, V. C. & Vaughan, M., unpublished observation.

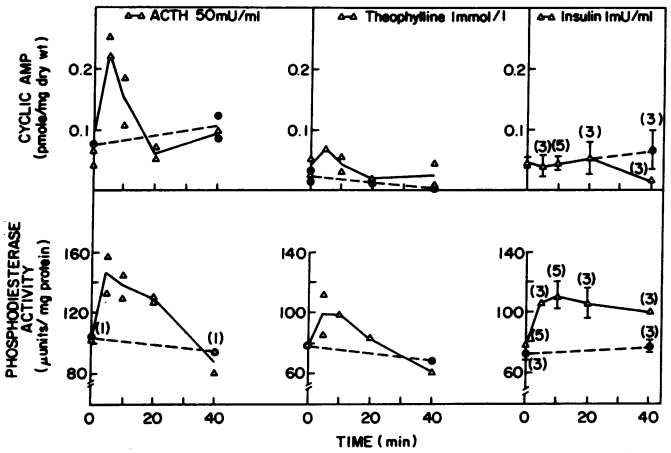

FIG. 2. Effects of ACTH, insulin, and theophylline on cyclic AMP content of fat cells and phosphodiesterase activity. Data from representative experiments with ACTH and theophylline are presented. A single point at one time denotes two values so similar that only one symbol could be used. Data from experiments with insulin are presented as the means ± indicated standard errors with the number of experiments in parentheses. In some instances the standard error is covered by the symbol. The SEM for zero time cyclic AMP content of 3 incubations with insulin represents also the data from 3 control incubations which were therefore omitted.

increases in phosphodiesterase activity at concentrations of 0.1 μM. In 10 experiments over a period of 6 months, incubation of cells with 2.7 μM epinephrine for 7.5 min increased phosphodiesterase activity 37 ± 3% (SEM), while in 15 other experiments the mean increment produced by 250 mU/ml ACTH was 44 ± 4%. When compared in the same experiment, phosphodiesterase activity was increased equally by maximally effective concentrations of ACTH or epinephrine or by both together (Table 3, Experiment 2).

Incubation of cells with 0.5 mM theophylline, also increased phosphodiesterase activity. The mean increment after 7.5 min was 41 + 5% (SEM) in 5 experiments. Doubling the concentration of theophylline did not increase its effect (Table 3, Experiment 3), and the addition of 0.5 mM theophylline in the presence of maximally effective concentrations of epinephrine or ACTH (or of both hormones together) did not further elevate phosphodiesterase activity (Table 3, Experiments 2–5).

After exposure of cells for 10 min to 2.7 μM epinephrine, washing 3 times in fresh medium (without epinephrine) over a period of 15 min completely reversed the effect on phosphodiesterase (data not shown). When the effect of epinephrine is terminated after 5 min by the addition of propranolol, the cell cyclic AMP content falls to basal levels in 1 to 2 min (2) and, as shown in Fig. 1, phosphodiesterase activity had reached basal levels in 2.5 min. Addition of ACTH at that time produced in 5 min a rise in phosphodiesterase activity equivalent to that initially produced by epinephrine in the same cells and to that produced by ACTH in cells not previously exposed to hormone.

Within 2 min after the addition of epinephrine, ACTH, or theophylline to fat cells, the phosphodiesterase activity was increased (data not shown); it reached a maximum usually by 5 min, and then declined, as shown in Fig. 2, for ACTH and theophylline. The changes in enzyme activity in all experi-

ments paralleled the changes in cell cyclic AMP and the decline in phosphodiesterase activity tended to lag behind the fall in cyclic AMP (Fig. 2).

Incubation of fat cells with insulin increases the activity of a phosphodiesterase that has a relatively high affinity for cyclic AMP (3) and is concentrated in a particulate fraction (4). As shown in Fig. 2, following the addition of insulin, phosphodiesterase activity was maximally increased within 5 min and remained elevated for at least 40 min, while the cyclic AMP content of the fat cells was not significantly changed. When phosphodiesterase activity was elevated by incubation of cells with ACTH (or epinephrine) plus theophylline (which would maintain the cell cyclic AMP content at a level higher than any of those shown in Fig. 2), insulin caused a further increase (Table 3, Experiments 4 and 5).

DISCUSSION

The transient rise in fat-cell phosphodiesterase activity produced by exposure of fat cells to epinephrine, ACTH, or theophylline is apparently confined to a portion of the enzyme that has a relatively high affinity for cyclic AMP and may be associated with plasma membranes (4). In the experiments reported here, ACTH caused a small but significant increase in K_m (from 0.39 to 0.45 μM cyclic AMP) and a 50% increase in V_{max} of this particulate phosphodiesterase. The cell fractions in which these effects were demonstrated contained relatively little "high K_m" phosphodiesterase activity and this was not altered by ACTH.

Following the addition of epinephrine, ACTH, or theophylline, the cyclic AMP content of fat cells rises rapidly and reaches a maximum in 4 to 7 min (2). The phosphodiesterase activity followed a similar course consonant with the possibility that the increase was secondary to accumulation of cyclic AMP, whether caused by stimulation of adenylate cyclase (ACTH, epinephrine) or inhibition of cyclic AMP degradation (theophylline). After 5 min, despite the continued presence of ACTH or theophylline, the cyclic AMP content of the cells and the phosphodiesterase activity both declined. When adenylate cyclase activity was abruptly reduced by the addition of propranolol to cells previously incubated with epinephrine, the cyclic AMP concentration fell rapidly to basal levels (2). In a similar experiment (see Fig. 1), propranolol likewise within 2.5 min abolished the stimulation of phosphodiesterase caused by epinephrine. Thus, under these conditions the activity of the particulate, high affinity phosphodiesterase appeared to change rapidly in parallel with and perhaps as a consequence of changes in cyclic AMP concentration.

As previously reported (4), incubation of fat cells with insulin also produces a rapid increase in the activity of a particulate, high affinity phosphodiesterase, but in the presence of insulin the enzyme activity remained elevated for at least 40 min and there was no demonstrable change in fat-cell cyclic AMP content. Insulin can prevent the rise in cyclic AMP caused by epinephrine or ACTH (2), and when theophylline is added along with the lipolytic hormone, insulin decreases cyclic AMP accumulation, although absolute levels remain elevated (1). Under the latter conditions, insulin still caused an increase in phosphodiesterase activity above that produced by ACTH plus theophylline. Whether the effects of insulin and ACTH (or epinephrine or theophylline) are exerted on the same or on different enzymes, the differences in time course and the fact that the effects of insulin and the lipolytic hormones are additive leaves little doubt that the underlying mechanisms are different.

It has been suggested (4, 8, 9) that insulin decreases fat cell cyclic AMP and interferes with lipolysis as a result of its effects on phosphodiesterase activity. On the other hand, the alterations in phosphodiesterase activity observed in the experiments with epinephrine and ACTH appear insufficient to explain the concomitant changes in cell cyclic AMP content with time. All of the data presented here, however, are consistent with the hypothesis that there is a component of the fat-cell phosphodiesterase whose activity can be acutely modified by the concentration of its substrate, cyclic AMP, and it seems probable that regulation of a similar type occurs also in other cells. We have recently demonstrated effects of ACTH and of theophylline on the cyclic AMP content of the specific cell fractions in which phosphodiesterase is assayed.§ Thus, we may be able to define more precisely the relationship between cyclic AMP concentration and enzyme activity and to determine whether cyclic AMP interacts directly with a regulatory moiety of the phosphodiesterase or acts indirectly, perhaps through a protein kinase.

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§ Lovell-Smith, C. J., Manganiello, V. C. & Vaughan, M., unpublished observations.