Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells

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Abstract Forskolin (7β-acetoxy-8, 13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one) induced both cyclic AMP production and lipolysis in intact fat cells, but stimulated lipolysis without increasing cyclic AMP at a concentration of 10^{-5}M. Homogenization of fat cells elicited lipolysis without elevation of cyclic AMP. Forskolin did not stimulate lipolysis in the homogenate. Forskolin stimulated both cyclic AMP production and lipolysis in a cell-free system consisting of endogenous lipid droplets and a lipoprotein lipase-free lipase fraction prepared from fat cells. However, at a concentration of 10^{-6}M, it induced lipolysis without increase in the cyclic AMP content in this cell-free system. In the cell-free system, homogenization of the lipid droplets resulted in marked increase in lipolysis to almost the same level as that with 10^{-4}M forskolin without concomitant increase in cyclic AMP. Addition of forskolin to a cell-free system consisting of homogenized lipid droplets and lipase did not stimulate lipolysis further. Phosphodiesterase activities were found to be almost the same both in the presence and absence of forskolin in these reaction mixtures. Although 10^{-3}M forskolin produced maximal concentrations of cyclic AMP: 6.7×10^{-7}M in fat cells and 2.7×10^{-7}M in the cell-free system, 10^{-4}M cyclic AMP did not stimulate lipolysis in the cell-free system. In a cell-free system consisting of lipid droplets and the lipase, pyrophosphate inhibited forskolin-induced cyclic AMP production, but decreased forskolin-mediated lipolysis only slightly. Based on these results, mechanism of lipolytic action of forskolin was discussed.—**Okuda, H., C. Morimoto, and T. Tsujita.** Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells. *J. Lipid Res.* 1992. **33:** 225–231.

Supplementary key words cell-free system

It is widely accepted that the rate-limiting step in lipolysis in fat cells is regulated by a cyclic AMP-dependent mechanism. The initial step in this process involves hormonal activation of adenylate cyclase, resulting in increased synthesis of cyclic AMP. This leads to activation of cyclic AMP-dependent protein kinase and subsequent phosphorylation and activation of hormone-sensitive lipase (1). Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one) is known to stimulate both adenylate cyclase activity and lipolysis in fat cells (2). It activates the catalytic subunit of adenylate cyclase directly (3). Despite the central role of the cyclic AMP content in hormonal-mediated lipolysis, cellular cyclic AMP contents are not always correlated with lipolytic rates (4, 5).

Mooney, Swicegood, and Marx (2) reported that increase in adenylate cyclase activity above a basal level required to activate lipolysis in response to forskolin was 5 times that with isoproterenol in both intact and permeabilized fat cells. They suggested that these results could be due to activation of lipolysis mediated by a β-adrenergic agent and its receptor independently of cyclic AMP. On the other hand, Allen (6) reported that the correlation between the responses of intracellular cyclic AMP and lipolysis to forskolin and to isoproterenol were very similar in intact fat cells.

In a previous study (7), we established a cell-free system in which epinephrine, DBcAMP, and theophylline stimulated lipolysis of endogenous lipid droplets from fat cells by lipase solution. In this system, these lipolytic agents did not induce lipolysis in the absence of added lipase. Recently, we found that forskolin also stimulated both cyclic AMP production and lipolysis in this cell-free system. The purpose of the present study was to examine forskolin-stimulated lipolysis in fat cells and the cell-free system with an emphasis on the role of cyclic AMP.

MATERIALS AND METHODS

Animals

Young male Wistar King rats, weighing 150 to 200 g, were given standard laboratory diet and water ad libitum. They were killed by cervical dislocation to minimize endogenous catecholamine secretion, and their epididymal adipose tissues were quickly removed.

Abbreviations: cyclic AMP or cAMP, 3′,5′-cyclic adenosine monophosphate; DBcAMP, dibutyryl cyclic adenosine monophosphate.

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Materials

Collagenase (type IV) was purchased from Worthington Corp. (Freehold, NJ). Glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase were from Amano Pharmaceutical Co. (Nagoya, Japan). Cyclic AMP and 5′-nucleotidase (from *Crotalus atrox* venom) were from Sigma (St. Louis, MO). Forskolin was from Nihon Kayaku Co. Ltd. (Tokyo, Japan) and heparin-Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine serum albumin was obtained from Wako Pure Chemical Industries (Osaka, Japan) and extracted by the method of Chen (8) to remove free fatty acid.

Methods

Measurement of lipolytic activity in fat cells. Isolated fat cells were obtained from rat epididymal adipose tissues by the method of Rodbell (9). The resultant fat cell fraction was divided into two portions. One portion (50 µl packed volume) was incubated for 1 h at 37°C in 225 µl of buffer A (25 mM TES, pH 7.4, containing 135 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$) supplemented with 2.5% bovine serum albumin and 25 µl of forskolin solution in buffer A. The other portion of fat cells was suspended in 225 µl of buffer A per 50 µl packed volume of cells supplemented with 2.5% bovine serum albumin, and homogenized in a Polytron homogenizer (Kinematica, GmbH, Switzerland). The homogenate was then incubated for 1 h at 37°C with 25 µl of forskolin solution in buffer A. After incubation, the reaction mixture was centrifuged (250 *g*, 5 min) at 4°C and the resultant supernatant was heated at 70°C for 10 min. Samples of 50 µl of the solution were incubated at 37°C for 5 min with 1 ml of 0.1 M HEPES buffer (pH 7.5) containing 2 mM ATP, 0.5 mM 4-aminoantipyrine, 1 mM EDTA, 0.5 U glycerol kinase, 4 U glycerol-3-phosphate oxidase, 2 U peroxidase, 2.7 mM *p*-chlorophenol, 0.04% Triton X-100, and 2 mM $MgSO_4 \cdot 7H_2O$. The glycerol content was then determined from the absorbance at 505 nm. In the assays of cyclic AMP content and phosphodiesterase activity, incubation was carried out for 30 min instead of 1 h and the reaction mixtures were used as sample solutions for these assays.

Preparation of endogenous lipid droplets. Isolated fat cells were obtained by the method of Rodbell (9), and 1 ml packed volume of cells was suspended in 4 ml of 5 mM Tris-HCl buffer (pH 7.4). The suspension was mixed by slowly inverting the centrifuge tube three times; it was then centrifuged at 200 *g* for 3 min at room temperature. The fat layer was mixed with 4 ml of 5 mM Tris-HCl buffer (pH 7.4) containing 0.025% Triton X-100 by slowly swinging the tube three times, and the mixture was centrifuged at 200 *g* for 3 min at room temperature. The fat layer was washed once with buffer A, incubated with buffer A at 37°C for 10 min, and centrifuged at 200 *g* for 3 min. Then, it was washed with buffer A. Lipase activity was removed from the fat layer by these procedures. Addition of epinephrine to the fat layer failed to stimulate lipolysis in the absence of added lipase (7). One g (dry weight) of the fat layer consisted of 870 µmol triglyceride, 0.71 µmol phospholipid, 0.52 µmol cholesterol, 342 µg carbohydrate, and 63 µg protein. Although the fat layer was a crude preparation, it was used as endogenous lipid droplets in this experiment.

Preparation of lipoprotein lipase-free lipase solution. Rat epididymal adipose tissue (0.7 g) was cut into small pieces with scissors and homogenized in 1 ml of buffer A (25 mM TES buffer, pH 7.4, containing 135 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$) in a Potter-Elvehjem homogenizer by five strokes by hand of a Teflon pestle. The homogenate was centrifuged (2,500 *g*, 15 min) at 10°C, and the resultant supernatant was applied to a heparin-Sepharose column (5 × 20 mm) equilibrated with buffer A to remove lipoprotein lipase. The unabsorbed fraction containing hormone-sensitive lipase was used as the lipase solution.

Estimation of diameter of endogenous lipid droplets. Fat cells, homogenized fat cells, lipid droplets, and homogenized lipid droplets were mixed with buffer A containing 2% osmium chloride. After standing for 3 h at 4°C, the preparations were subjected to scanning electron micrography (Hitachi H-500). The diameters of lipid droplets were estimated with these electron micrographs by an image analyzer.

Lipolysis in a cell-free system consisting of lipase and endogenous lipid droplets. A sample of 25 µl of packed endogenous lipid droplets was incubated at 37°C for 1 h with 100 µl of lipase solution containing hormone-sensitive lipase, 25 µl of forskolin solution, 100 µl of buffer A containing 2.5% bovine serum albumin, and 25 µl of buffer A. Another sample of 25 µl of lipid droplets was homogenized in 100 µl of buffer A containing 2.5% bovine serum albumin and 25 µl of buffer A in a Polytron homogenizer. Then 25 µl of forskolin and 100 µl of the lipase were added to the homogenate, and the mixture was incubated as described above. The reaction was stopped by immersing the polyethylene tube containing the reaction mixture in ice. The reaction mixture was centrifuged (250 *g*, 5 min) at 4°C and the resultant supernatant was heated at 70°C for 10 min. A sample of 50 µl of the supernatant was incubated at 37°C for 5 min with 1 ml of 0.1 M HEPES buffer (pH 7.5) containing 2 mM ATP, 0.5 mM 4-aminoantipyrine, 1 mM EDTA, 0.5 U glycerol kinase, 4 U glycerol-3-phosphate oxidase, 2 U peroxidase, 2.7 mM *p*-chlorophenol, 0.04% Triton X-100, and 2 mM $MgSO_4$, and then its glycerol content was deter-

mined by measuring the absorbance at 505 nm. Previously, we reported that epinephrine-induced lipolysis proceeded linearly at least until 800 nmol/mg protein per h of glycerol was produced in the cell-free system consisting of lipase and intact lipid droplets (10). In the assays of cyclic AMP content and phosphodiesterase activity, incubation was carried out for 30 min instead of 1 h and the reaction mixtures were used as sample solutions for these assays.

Assay of cyclic AMP content. All the sample solutions were homogenized at 4°C with 6% TCA to obtain 1-ml volumes of 10% (v/v) homogenate. These homogenates were used to estimate the cyclic AMP content with a radioimmunoassay kit from Daiichi Kagaku Co. (Tokyo, Japan).

Assay of phosphodiesterase. Phosphodiesterase activity was determined by the method of Kono, Robinson, and Sarver (11). All sample solutions were homogenized just after incubation. The homogenate was centrifuged at 1,500 *g* for 10 min at 4°C and the resultant supernatant fraction was used as enzyme solution. One hundred µl of the enzyme solution was incubated with 100 nM cyclic [^{3}H]AMP (approximately 10 nCi) in the presence of 4 mM $MgCl_2$ and 33 mM Tris-HCl buffer, pH 7.4, in a total volume of 0.25 ml. The incubation was carried out at 30°C for 10 min and terminated by the addition of 0.1 ml of 0.1 N HCl. The incubated solution was mixed with 0.05 ml of unlabeled 5 mM AMP plus 5 mM cyclic AMP, placed in a water bath at 70°C for 4 min, cooled, and neutralized with 0.1 ml of 0.1 N NaOH. The neutralized solution was supplemented with 0.6 U of 5′-nucleotidase in 0.05 ml of 0.1 M Tris-HCl buffer (pH 8.0), and incubated at 37°C for 20 min. The incubation was terminated by the addition of 0.05 ml of 200 mM sodium EDTA containing 5 mM adenosine (pH 7.0). Subsequently, an aliquot (0.5 out of 0.6 ml) of the reaction mixture was withdrawn and applied to a column (5 × 30 mm) of AG 1-X2 (200 to 400 mesh, in chloride form, from Bio-Rad) that had been washed with water. The loaded column was eluted with water. The first 1.15 ml of the effluent (including the initial 0.5 ml replaced by the sample solution) was discarded, and the next 2.5 ml was collected. The radioactivity in the latter (adenosine) fraction was determined by a conventional liquid scintillation counting method. In this assay, each sample was assayed routinely in triplicate and the mean value of the three results was counted as n = 1 in statistical analyses.

Analysis of data. Student's *t* test was used to determine the significance of differences.

RESULTS

Effect of forskolin on fat cells and homogenates of fat cells

Forskolin induced lipolysis at concentrations of over 10^{-5}M in intact fat cells, but not at 10^{-6}M (**Fig. 1**). Homogenization of fat cells resulted in lipolysis in the absence of forskolin, which was not affected by the addition of forskolin (Fig. 1). Forskolin increased the cyclic AMP content of intact fat cells at concentrations of 10^{-4}M or more, but not at the concentration of 10^{-5}M, which induced lipolysis (**Fig. 2**). In the homog-

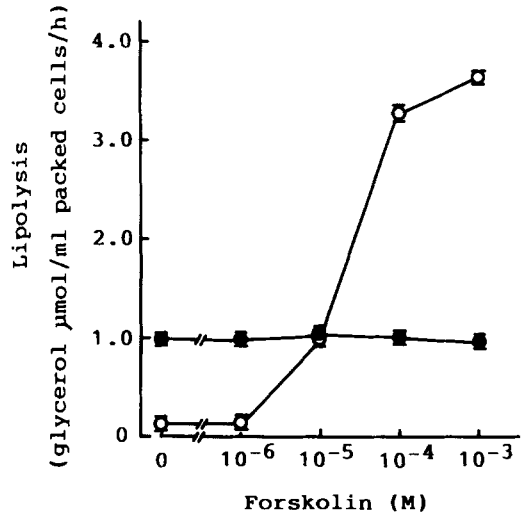

Fig. 1. Lipolysis induced by forskolin in fat cells and homogenates of fat cells. Incubation was carried out for 1 h; (○), intact fat cells; (●), homogenized fat cells. Each point represents the mean ± SE of four separate assays.

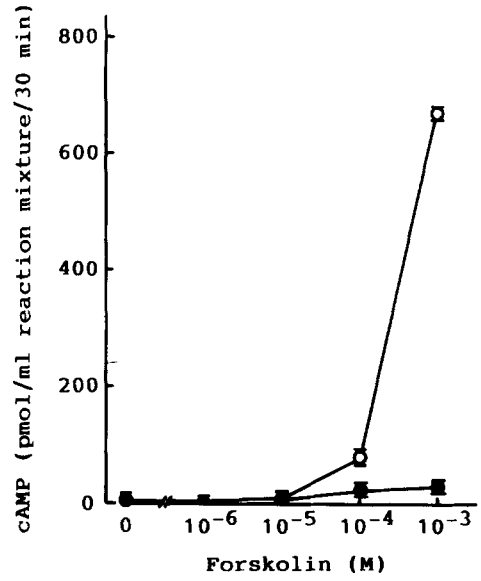

Fig. 2. Effect of forskolin on the cyclic AMP content of fat cells and homogenates of fat cells. Incubation was carried out for 30 min; (○), intact fat cells; (●), homogenized fat cells. Each point represents the mean ± SE of three separate assays.

enate, the cyclic AMP content was nearly zero with 10^{-5}M forskolin and slightly increased by concentrations over 10^{-4}M. Homogenization of the fat cells did not increase the cyclic AMP content (Fig. 2). The average diameter of lipid droplets in fat cells was found to be 49.4 ± 0.7 μm, while it reduced to 22.6 ± 0.2 μm after homogenization of fat cells.

Effect of forskolin on lipolysis in the cell-free system

In contrast to its effect on intact fat cells, 10^{-6}M forskolin stimulated lipolysis in a cell-free system containing endogenous lipid droplets and lipoprotein lipase-free lipase (**Fig. 3**). When forskolin was incubated with endogenous lipid droplets in the absence of the lipase at its concentrations of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}M, no significant lipolysis was found (data not shown). Homogenization of the lipid droplets markedly increased lipolysis in the cell-free system, and forskolin did not stimulate this lipolysis further (Fig. 3).

In the cell-free system consisting of intact lipid droplets and the lipase, the cyclic AMP content was increased by 10^{-5}M forskolin, but not by 10^{-6}M forskolin, which stimulated lipolysis (**Fig. 4**). Furthermore, homogenization of the lipid droplets failed to elevate the cyclic AMP content, although it markedly increased lipolysis, and forskolin did not increase the cyclic AMP content in this system (Fig. 4). The average diameter of intact lipid droplets was 56.6 ± 0.7 μm, while it reduced to 33.9 ± 0.9 μm after homogenization of the intact lipid droplets.

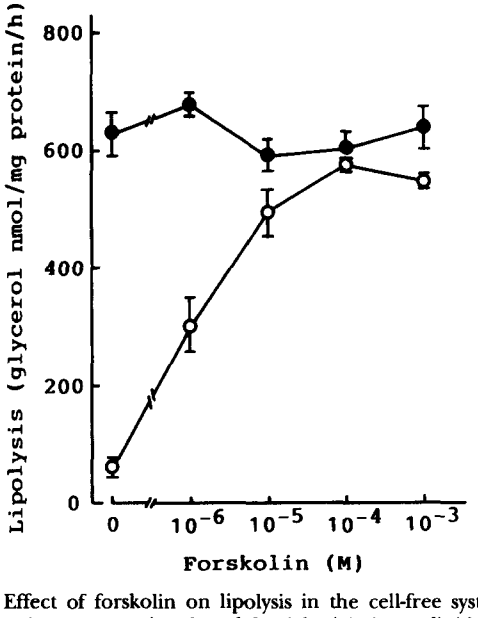

Fig. 3. Effect of forskolin on lipolysis in the cell-free system. The reaction mixtures were incubated for 1 h; (○), intact lipid droplets; (●), homogenized lipid droplets. Each point represents the mean ± SE of four separate assays.

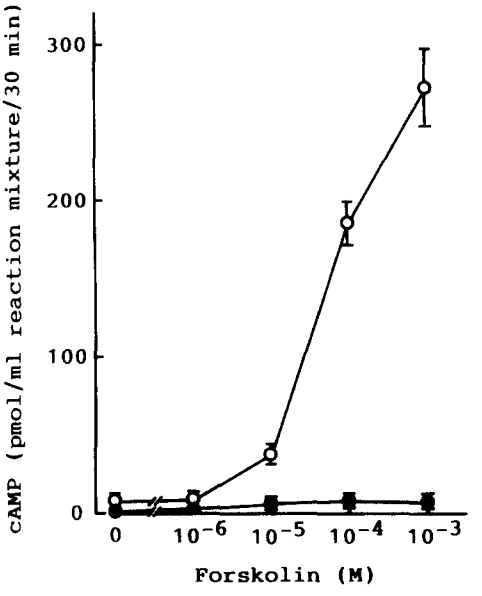

Fig. 4. Effect of forskolin on the cyclic AMP content in the cell-free system. Incubation was carried out for 30 min; (○), intact lipid droplets; (●), homogenized lipid droplets. Each point represents the mean ± SE of three separate assays.

It is well known that cyclic AMP contents are influenced by phosphodiesterase activities in these preparations. Therefore, we tried to estimate phosphodiesterase activities in fat cells, homogenized fat cells, and the cell-free system consisting of the lipase and intact lipid droplets or homogenized lipid droplets. After incubation for 30 min, the reaction mixtures were immediately homogenized and centrifuged. The resultant supernatant fraction was subjected to estimation of phosphodiesterase activity as described in Materials and Methods.

As shown in **Table 1,** phosphodiesterase activities were found to be almost the same both in the presence and absence of forskolin in each reaction mixture.

In fat cells and the cell-free system, 10^{-3}M of forskolin produced 6.7×10^{-7}M and 2.7×10^{-7}M of cyclic AMP, respectively (Figs. 2 and 4). On the other hand, higher concentrations of cyclic AMP (10^{-6}, 10^{-5}, and 10^{-4}M) failed to significantly stimulate lipolysis in the cell-free system as shown in **Table 2.**

Pyrophosphate inhibits adenylate cyclase activity (12). As shown in **Fig. 5,** addition of pyrophosphate considerably reduced the cyclic AMP content induced by 10^{-4}M forskolin in the cell-free system consisting of lipid droplets and the lipase, but decreased forskolin-induced lipolysis only slightly; at a concentration of 5 mM, it reduced forskolin-induced cyclic AMP production about 80% but lipolysis by only 18.6% (Fig. 5).

TABLE 1. Phosphodiesterase activities in various reaction mixtures

Forskolin (M)	Phosphodiesterase Activity (cAMP pmol/ml enzyme/min)			
			Cell-Free System	
	Intact Fat Cells	Homogenized Fat Cells	Intact Lipid Droplets	Homogenized Lipid Droplets
0	0.52	0.56	0.86	0.75
10^{-6}	0.58	0.60	0.77	0.73
10^{-5}	0.51	0.56	0.83	0.72
10^{-4}	0.57	0.57	0.85	0.75
10^{-3}	0.55	0.57	0.85	0.70

The cell-free system consisted of the lipase and intact lipid droplets or homogenized lipid droplets. The procedures are as described in Materials and Methods. Each value is the mean of three assays.

DISCUSSION

In the present investigation, we examined the effects of forskolin on lipolysis and cyclic AMP production in fat cells and a cell-free system consisting of endogenous lipid droplets from fat cells and lipase containing hormone-sensitive lipase.

In the cell-free system, epinephrine, DBcAMP, and theophylline induce lipolysis in the presence, but not the absence of added lipase (7). Forskolin stimulated lipolysis in this cell-free system, but its effective concentration for lipolysis was different from that for intact fat cells: 10^{-6}M forskolin significantly induced lipolysis in the cell-free system, but not in fat cells (Figs. 1 and 3). Forskolin also increased the cyclic AMP content in the cell-free system (Fig. 4). We examined the kinetics of induction of cyclic AMP production by 10^{-4}M forskolin, finding that in both fat cells and the cell-free system the induction was maximal after 30 min. Therefore, we measured forskolin-induced cyclic AMP production after incubation for 30 min.

Forskolin had no appreciable effect on cyclic AMP production by either lipid droplets or the lipase. Furthermore, it did not induce cyclic AMP production in a mixture of heat (100°C, 10 min)-treated lipid droplets and the lipase. On the other hand, it induced cyclic AMP production in a mixture of intact lipid droplets and heat-treated lipase (data not shown). These results suggest that lipid droplets contain adenylate cyclase and that its substrate, ATP, was included in the lipase fraction.

TABLE 2. Effect of cyclic AMP on lipolysis in the cell-free system

cAMP (mM)	Lipolysis (glycerol nmol/mg protein/h)
0	12 ± 4
10^{-6}	19 ± 5
10^{-5}	26 ± 7
10^{-4}	29 ± 4

Experimental conditions are described in Materials and Methods. Values are means ± SE for four separate assays.

We found that 10^{-5}M forskolin stimulated lipolysis in fat cells, but did not increase their cyclic AMP content (Figs. 1 and 2). Similarly, in the cell-free system, 10^{-6}M forskolin induced lipolysis without concomitant increase in cyclic AMP (Figs. 3 and 4). Phosphodiesterase activities were found to remain constant both in fat cells and the cell-free system, suggesting that the rate of hydrolysis of cyclic AMP was almost the same in these concentrations of forskolin (Table 1). Furthermore, although the maximal concentrations of cyclic AMP induced by 10^{-3}M forskolin were found to be 6.7×10^{-7}M in fat cells and 2.7×10^{-7}M in the cell-free system, respectively, 10^{-4}M cyclic AMP did not significantly stimulate lipolysis in the cell-free system (Table 2).

The most striking finding in the present study was the effect of homogenization of lipid droplets or fat cells on lipolysis and the content of cyclic AMP;

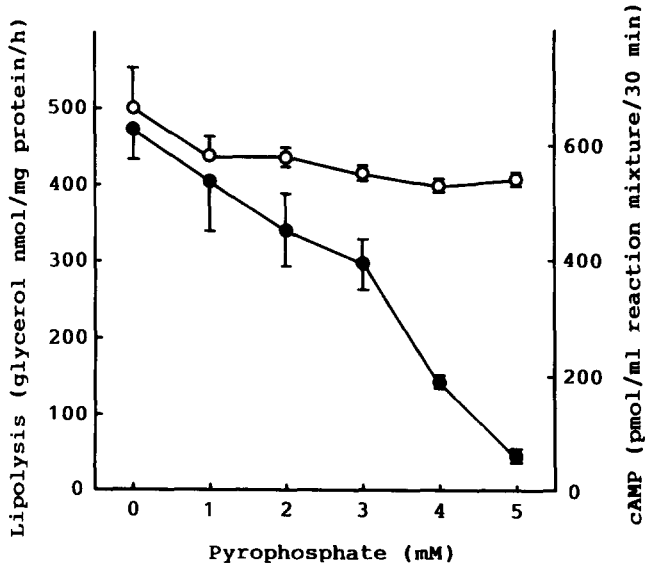

Fig. 5. Effect of pyrophosphate on forskolin-induced lipolysis and the cyclic AMP in the cell-free system. Lipolysis and the cyclic AMP content were examined with 10^{-4}M forskolin in the cell-free system consisting of lipid droplets and the lipase. Pyrophosphate was added with 25 µl of buffer A; (○), lipolysis; (●), cyclic AMP content.

homogenization of lipid droplets in the cell-free system resulted in marked increase in lipolysis to a level corresponding to the maximum rate of lipolysis induced by forskolin (Fig. 3), but the cyclic AMP level remained nearly zero (Fig. 4).

Homogenization of fat cells also resulted in lipolysis without increase in cyclic AMP (Figs. 1 and 2). Furthermore, pyrophosphate considerably inhibited forskolin-induced cyclic AMP production in the cell-free system, but reduced forskolin-induced lipolysis only slightly (Fig. 5). All these results indicate that cyclic AMP content is not correlated with lipolysis.

Previous reports have also pointed out the inconsistencies observed when intracellular cyclic AMP and its related enzyme are correlated with lipolysis. Mooney et al. (2) demonstrated that the adenylate cyclase activity required to reach maximum lipolysis was higher for forskolin than for isoproterenol in digitonin-permeabilized adipocytes (2). A similar observation was made by Litosch et al. (13) on intact adipocytes and was cited as suggesting compartmentation of intracellular cyclic AMP. On the other hand, Allen (6) reported that the correlation between intracellular cyclic AMP and lipolysis was very similar in response to forskolin and isoproterenol in intact cells. If there is compartmentation of cyclic AMP, it would not be surprising that under some conditions intracellular cyclic AMP is correlated with lipolysis, while under others it is not. In any event, it remains to be demonstrated whether compartmentation of cyclic AMP is involved in regulation of lipolysis in adipocytes.

The present study demonstrated that forskolin failed to stimulate lipolysis after homogenization of fat cells or the lipid droplets in the cell-free system. The diameters of the lipid droplets in fat cells and the isolated lipid droplets were found to be reduced after homogenization. These results suggest that the physico-chemical character of the intact lipid droplets (for example, the surface phospholipid content) may play an important role in forskolin-induced lipolysis in fat cells and the cell-free system (7).

In 1966, Okuda, Yanagi, and Fujii (14) first demonstrated the existence of another mechanism in which epinephrine did not act on lipase but on endogenous lipid droplets, the substrate of the enzyme (14–21). Mosinger (22) confirmed the existence of this new type of mechanism. Wise and Jungas (23) also reported that incubation of epinephrine with rat adipose tissue resulted in activation of the endogenous substrate of lipase, and proposed another lipolytic mechanism besides that of activation of hormone-sensitive lipase through cyclic AMP-dependent protein kinase. Oschry and Shapiro (24) presented evidence that the availability of endogenous substrate might be the major factor in stimulation of the activity of lipase in fat cells. Recently, we suggested that the site of the lipolytic action of catecholamine may be the surface of endogenous lipid droplets in fat cells (7, 10, 25). In the present investigation, it was demonstrated that cyclic AMP content was not correlated with forskolin-induced lipolysis in fat cells and the cell-free system consisting of the lipase and the lipid droplets. Furthermore, it was found that forskolin did not stimulate lipolysis in homogenized fat cells and the cell-free system with homogenized lipid droplets instead of intact ones. Homogenization caused reduction of the diameters of lipid droplets in fat cells and the isolated lipid droplets.

These current data and those cited above do not rule out the possibility that forskolin-induced lipolysis may not be mediated by a cyclic AMP-dependent process but by another one including endogenous lipid droplets in fat cells. Further experiments are needed to clarify the mechanism of the lipolytic action of forskolin.

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