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RATE-LIMITING STEPS IN ISOPROTERENOL AND FORSKOLIN STIMULATED LIPOLYSIS

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Abstract—Using the flask-incubated fat cell system, effects of isoproterenol and forskolin on glycerol release, cyclic AMP levels and protein kinase were studied. Isoproterenol increased cyclic AMP levels, protein kinase activity and glycerol release over the same concentration range (10^{-9} M to 10^{-6} M). Forskolin also increased all three variables in a concentration-dependent manner (10^{-7} M to 10^{-4} M). The maximum response for each variable was significantly greater with forskolin than with isoproterenol. A combination of isoproterenol and forskolin resulted in an additional increase in cyclic AMP over forskolin alone, but no significant increase in protein kinase activity or glycerol release. These results support the concepts that the maximum lipolytic response to isoproterenol is limited by the accumulation of cyclic AMP and the maximum lipolytic response to forskolin is limited by some step distal to cyclic AMP production, possibly activation of protein kinase. At high concentrations of forskolin or with a combination of forskolin and isoproterenol, cyclic AMP levels were in excess of those needed to maximally activate protein kinase and lipolysis.

It is generally agreed that the lipolytic activity of a wide variety of hormones is secondary to the activation of adenylate cyclase with the subsequent increase in intracellular levels of cyclic AMP [1]. Cyclic AMP activates a protein kinase which, in turn, phosphorylates and activates a triglyceride lipase. The lipase catalyzes the breakdown of triglyceride to free fatty acids and glycerol [1].

Although much information is available on each individual reaction in the lipolytic sequence, little is known about the quantitative relationship of these reactions. Reports by Butcher and Baird [2] and Fain [3] suggest that maximum changes in lipolytic rates occur with very small changes in cyclic AMP levels and that the accumulation of cyclic AMP in the tissue is many-fold higher that that necessary to activate the lipolytic process. These results were obtained by measuring cyclic AMP accumulation in the presence of a phosphodiesterase inhibitor and the lipolytic rates in the absence of such compounds, making interpretations difficult. Soderling *et al.* [4] described the effects of hormones on adipose tissue protein kinase activity ratios and correlated these changes with changes in cyclic AMP content. A close correlation was found between cyclic AMP levels and protein kinase activity; however, these changes did not correlate as well with lipolytic rates.

Using the perifused fat cell system, Sengupta *et al.* [5] compared changes in cyclic AMP, protein kinase activity and lipolytic rates following isoproterenol stimulation. Linear correlations between cyclic AMP levels and protein kinase, and between cyclic AMP and lipolytic rates, were found over approximately a 6-fold concentration range of cyclic nucleotide.

The present paper describes the quantitative relationships among cyclic AMP levels, protein kinase activity, and lipolytic activity in flask-incubated fat cells. Isoproterenol, a well known beta-adrenergic receptor agonist, was studied as was the diterpine derivative forskolin. Forskolin has been shown to activate adenylate cyclase in intact cells by a direct action on the catalytic subunit [6].

MATERIALS AND METHODS

Experiments were carried out on fed, Sprague–Dawley rats weighing 200–300 g. Fat pads were removed, and isolated fat cells were prepared by the method of Lech and Calvert [7].

Fat cell incubation. An aliquot of fat cells (approximately 60 mg dry weight) was placed in a plastic flask with Krebs–Ringer–phosphate buffer (pH 7.4) containing 4% (w/v) bovine serum albumin. Temperature was maintained at 37° with a shaking water bath. Drugs were added and incubations were carried out for 45 min except in the time-course experiments. The final volume was 3.0 ml.

Following incubation, the contents of the flask were transferred to a handheld glass homogenizer containing 1 ml of a solution of isobutyl methyl xanthine (0.4 mM), EDTA (40 mM), and NaCl (2.0 M) at 0°. The cells were homogenized with three strokes of a motor-driven pestle. An aliquot of the homogenate was taken for dry weight [5], and another aliquot was added to an equal volume of 10% (w/v) trichloroacetic acid for assay of cyclic AMP by the radioimmunoassay of Harper and Brooker [8]. The remainder of the homogenate was centrifuged for 10 min at 0° (2000 *g*), and the fat cake was removed. Protein kinase activity was assayed by the method of Reimann *et al.* [9]. Glycerol content was assayed by the fluorometric method of Chernick [10].

Statistics. Levels of significance were determined by Student's *t*-test for paired data.

Materials. *l*-Isoproterenol, cyclic AMP, bovine serum albumin (fraction V), glycerol kinase, type II-

A histone and adenosine 5′-monophosphate were purchased from the Sigma Chemical Co. (St. Louis, MO). All nucleotides, sugar phosphates and enzymes used for the [α-^{32}P]ATP synthesis [11] were from Boehringer Mannheim (New York, NY). Carrier-free [^{32}P] inorganic phosphate was purchased from the ICN Corp. (Irvine, CA). Adenosine 3′:5′-cyclic phosphoric acid [^{125}I]-2′-*O*-succinyl iodotyrosine methylester was purchased from the New England Nuclear Corp. (Boston, MA) and antiserum to cAMP from Janus Laboratories (El Cajon, CA). Forskolin was purchased from the Calbiochem-Bering Corp. (LaJolla, CA).

RESULTS

Time-course of cyclic AMP accumulation, protein kinase activation and glycerol release. Following the addition of isoproterenol (3.3×10^{-7} M), there was a time-dependent increase in cyclic AMP content, activation of protein kinase, and release of glycerol (Fig. 1). Both cyclic AMP levels and protein kinase activity reached maximum levels by 15 min and remained at that level up to 45 min. Glycerol release increased slightly at 10 min of isoproterenol exposure and reached maximum rates by 20 min. This maximum rate was maintained for at least an additional 25 min.

Similar results were obtained when forskolin (3.3×10^{-6} M) was added to the fat cells (Fig. 2). Cyclic AMP levels reached maximum levels between 10 and 15 min fell slightly at 30 min and then remained constant through 60 min of incubation. Protein kinase activity reached maximum value between 20 and 30 min, fell slightly at 45 min and remained at that level through 60 min of incubation. Glycerol release increased at 10 min and had reached maximum rates of release by 15 min. The maximum rate of release remained constant through 60 min of incubation.

Dose–response relationship. In dose–response experiments, samples were collected after 45 min of incubation with either isoproterenol or forskolin, and the results are shown in Fig. 3. Significant ($P < 0.05$) increases in cyclic AMP were seen at isoproterenol concentrations of 10^{-8} M and above. Forskolin concentrations at 10^{-6} M and above resulted in significant ($P < 0.05$) elevations in cyclic AMP. An increase of nearly 25-fold was seen with the highest concentration of forskolin while the maximum response to isoproterenol was approximately 2-fold.

Protein kinase activity was increased by both isoproterenol and forskolin. Significant ($P < 0.05$) increases were seen with concentrations of isoproterenol of 10^{-8} M and above. Forskolin produced significant ($P < 0.05$) increases in protein kinase activity at concentrations of 10^{-6} M to 10^{-4} M.

Glycerol release was increased by isoproterenol at concentrations of 3.3×10^{-9} M and higher ($P < 0.05$) and by forskolin at concentrations of 10^{-6} M and higher ($P < 0.05$). The maximum response to forskolin was about two times the maximum response to isoproterenol.

Maximum effective concentrations of isoproterenol and forskolin were added separately and in combination to the same population of fat cells (Table 1). The responses to forskolin were significantly ($P < 0.05$) greater than those to isoproterenol with all variables measured. The combination of isoproterenol and forskolin resulted in a greater response in cyclic AMP levels but not in protein kinase activity or glycerol release.

DISCUSSION

The lipolytic activity of a variety of agents is thought to be the result of an increase in cyclic AMP levels with the subsequent activation of cyclic AMP-dependent protein kinase. This enzyme, in turn,

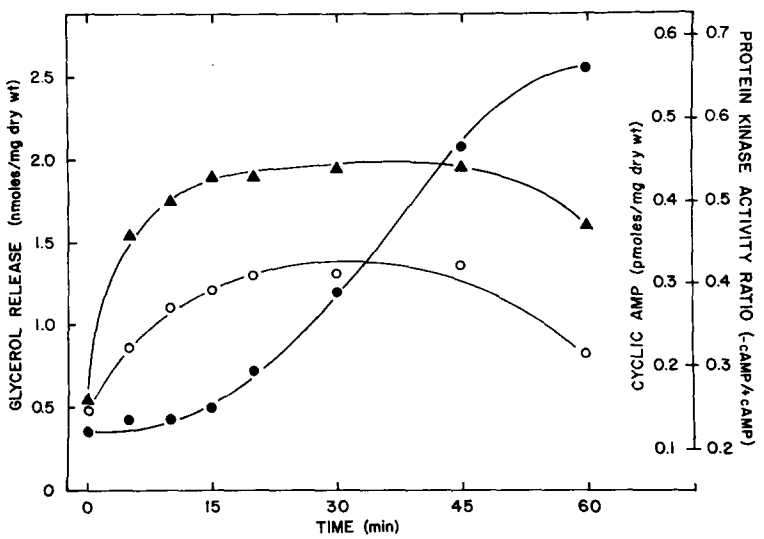

Fig. 1. Time-course for the effects of isoproterenol. Fat cells were incubated with isoproterenol (3.3×10^{-7} M) for various times following which samples were taken for assay of glycerol (●) protein kinase activity (▲) and cyclic AMP levels (○). Each point is the mean of three experiments.

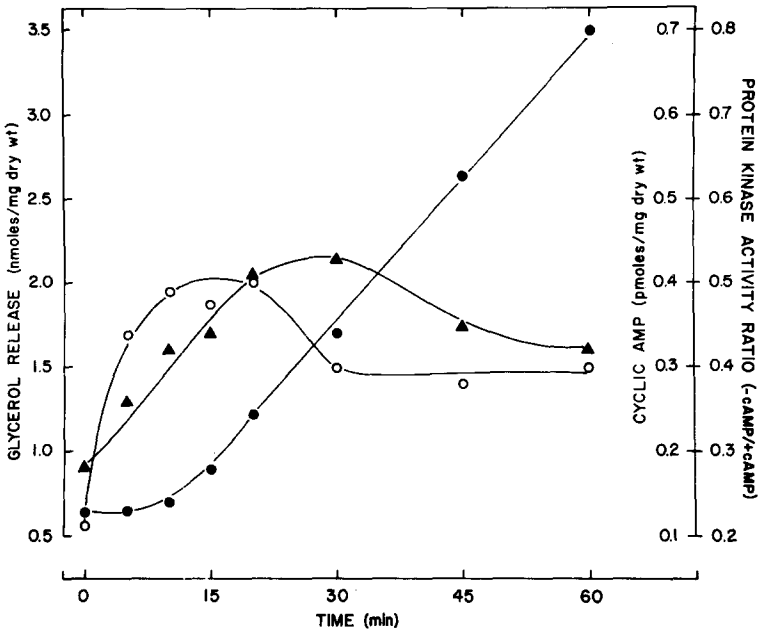

Fig. 2. Time-course for the effects of forksolin. Fat cells were incubated with forksolin (3.3×10^{-6} M) for various times following which samples were taken for assay of glycerol (●), protein kinase activity (▲), and cyclic AMP levels (○). Each point is the mean of four experiments.

phosphorylates and thus activates a triglyceride lipase which catalyzes the breakdown of triglyceride to free fatty acids and glycerol [1].

Although the individual steps in this sequence of reactions have been identified, considerable question remains about the quantitative relationship between each of these steps. Butcher and Baird [2] and Fain [3] have reported that maximum changes in lipolytic rates occur with very small changes in cyclic AMP levels when epinephrine is used as the lipolytic stimulant. These authors further observed that the accumulation of cyclic AMP in the tissue is many-fold higher than that necessary to maximally activate the lipolytic process. This suggested that, with agents that stimulated the beta-adrenergic receptor, the rate-limiting step in the lipolytic process was distal to the accumulation of cyclic AMP.

Results presented here demonstrate that isoproterenol, a beta-adrenergic agonist, increased cyclic AMP levels, protein kinase activity, and glycerol release over the same concentration range. A 2- to 3-fold increase in cyclic AMP levels related to an increase of approximately 2-fold in protein kinase activity, and approximately a 20-fold increase in glycerol release. No evidence was found for an amount of cyclic AMP in excess of that needed to maximally stimulate lipolysis. Additionally, at a concentration of isoproterenol that maximally activated lipolysis, protein kinase activity was not rate limiting, being at approximately 55% of its maximum activity. These data demonstrate that the maximum lipolytic response to isoproterenol was limited by the ability of the drug to increase cyclic AMP levels and not by some step distal to cyclic AMP accumulation.

Forskolin stimulated lipolytic activity to a level twice that of isoproterenol. This was the result of an increase in cyclic AMP levels of nearly ten times that of isoproterenol and of protein kinase activation of

Table 1. Effects of isoproterenol and forskolin on cyclic AMP levels, protein kinase activity and glycerol release*

Condition	Cyclic AMP (pmoles/mg dry wt)	Protein kinase (−cAMP/+cAMP)	Glycerol release (μmoles/45 min/mg dry wt)
Control	0.31 ± 0.04	0.40 ± 0.05	0.10 ± 0.10
Isoproterenol (10^{-6} M)	0.72 ± 0.15†	0.71 ± 0.06†	3.62 ± 1.05†
Forskolin (1.34×10^{-4} M)	6.50 ± 0.84†‡	1.01 ± 0.04†‡	7.54 ± 0.45†‡
Isoproterenol + forskolin	18.37 ± 4.36†‡§	0.96 ± 0.04†‡	6.75 ± 1.02†‡

* Values are expressed as mean ± S.E.M. for five experinents.
† $P < 0.05$ as compared to control.
‡ $P < 0.05$ as compared to isoproterenol.
§ $P < 0.05$ as compared to forskolin.

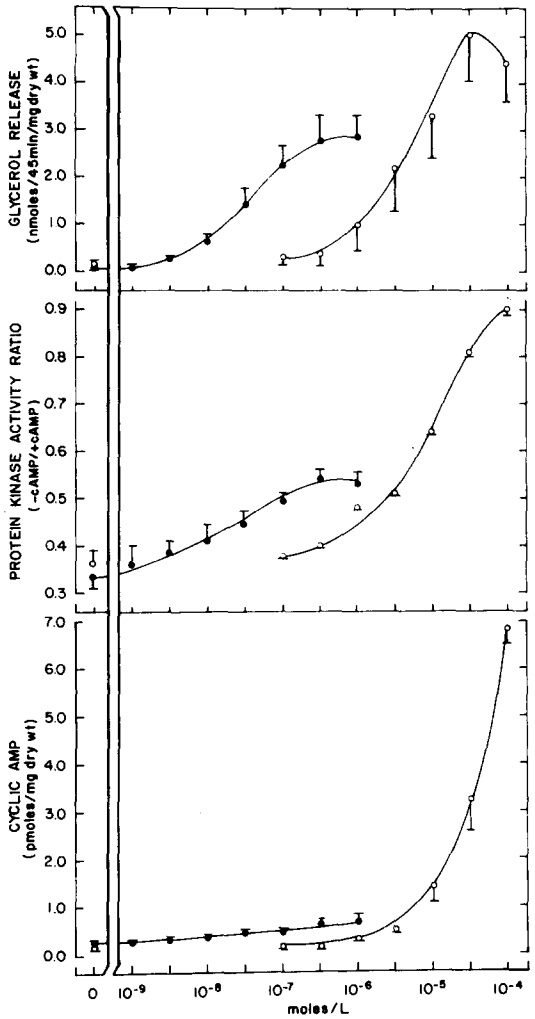

Fig. 3. Dose–response relationships for drugs and glycerol release (top), protein kinase activity (middle), and cyclic AMP levels (bottom). Fat cells were incubated with isoproterenol (●) or forskolin (○) for 45 min, following which samples were taken for assay. Each point represents the mean ± S.E.M. for five (protein kinase activity) or seven (cyclic AMP levels and glycerol release) experiments.

near twice that of isoproterenol. These data suggest that, at high concentrations of forskolin, activation of protein kinase was the rate-limiting step in the lipolytic process. Under these conditions protein kinase activity was at or near the theoretical maximum and thus rate limiting. This conclusion is supported by the observation that increasing the level of cyclic AMP in the cell with a combination of forskolin and isoproterenol did not result in any additional activation of protein kinase activity nor in higher rates of lipolysis. Thus, some step distal to cyclic AMP was rate limiting, most likely protein kinase activation.

The range of concentrations of forskolin which increase cyclic AMP levels and glycerol release reported in this study is similar to that reported by Litosch *et al.* [12]. However, these authors reported that no difference was seen between the maximum lipolytic response to forskolin and isoproterenol, whereas in the present study forskolin activated lipolysis to a level twice that of isoproterenol. Possible explanations for the differences include differences in the anatomical source of the adipose tissue, and the weight and sex of the animals.

The maximum response in glycerol release and protein kinase activity with isoproterenol correlated with a 2-fold increase in cyclic AMP. Previous work from this laboratory using the perifused fat cell system demonstrated that a 6-fold increase in cyclic AMP levels was necessary for full expression of the lipolytic and protein kinase response to isoproterenol [5]. The differences may be the result of the two different model systems. In the perifused fat cell system, the cells are constantly washed with fresh buffer thus preventing the build-up of metabolites which can accumulate with the flask incubation method.

In summary, the results reported here support the concept that the maximal lipolytic response to isoproterenol is limited by the accumulation of cyclic AMP within the fat cell and the maximum lipolytic response to forskolin is limited by the activation of protein kinase. At concentrations of the two drugs that produce the same accumulation of cyclic AMP, similar protein kinase activities resulted. At high concentrations of forskolin or with a combination of forskolin and isoproterenol, cyclic AMP levels were in excess of those needed to maximally activate protein kinase and lipolysis.

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