Stimulation by Growth Hormone and Dexamethasone of Labeled Cyclic Adenosine 3', 5'-Monophosphate Accumulation by White Fat Cells*

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SUMMARY

The accumulation of cyclic adenosine 3', 5'-monophosphate (cyclic AMP) was determined by measuring the accumulation of labeled cyclic AMP in fat cells whose adenine nucleotides had been labeled with adenine-3H during the isolation of the white adipose cells. The addition of growth hormone and 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione (dexamethasone) increased the accumulation of labeled cyclic AMP and lipolysis after a lag period of at least 1 hour. At the end of 4 hours of incubation, most of the increase in accumulation of cyclic AMP due to epinephrine or growth hormone and dexamethasone was in the medium rather than in the cells. Puromycin and cycloheximide, which are inhibitors of protein synthesis, blocked the increases in both labeled cyclic AMP accumulation and lipolysis due to growth hormone and dexamethasone. However, the addition of cycloheximide alone did stimulate cyclic AMP accumulation without affecting lipolysis due to theophylline. Growth hormone and glucocorticoid also increased phosphorylase activity in fat cells, and this was blocked by cycloheximide. These results indicate that there is an increase in accumulation of labeled cyclic AMP due to growth hormone and glucocorticoid which is blocked by inhibitors of protein synthesis.

EXPERIMENTAL PROCEDURE

White fat cells were isolated from the parametrial and peri-ovarian adipose tissue of immature female rats (Charles River CD strain). The rats (130 to 150 g) were maintained on Purina laboratory chow ad libitum. Fat cells were isolated by the procedure of Rodbell (11) from the pooled adipose tissue of three to four rats by digestion in 6 to 8 ml of phosphate buffer containing 4% albumin, 0.5 mg per ml of crude bacterial collagenase (Clostridium histolyticum, Worthington), and 1 mg per ml of crystalline trypsin (Worthington). Cells were isolated by digestion with trypsin in addition to collagenase to diminish the effects of insulin or "insulin-like" substances on the response to lipolytic agents (12). Cells isolated by digestion with trypsin plus collagenase respond normally to lipolytic agents such as epinephrine, theophylline, and growth hormone plus dexamethasone, but the response to insulin is abolished (12).

No glucose was present during either the isolation or the incubation of the cells, but 0.1 mg per ml of trypsin inhibitor (egg white ovomucoid) was present during incubation of the cells. The phosphate buffer contained: NaCl, 120 mM; KCl, 5.15 mM; CaCl₂, 1.4 mM; MgSO₄, 1.4 mM; Na₂HPO₄, 10 mM; and 4% bovine Fraction V albumin (Pentex, Kankakee, Illinois). The buffer was made up fresh daily and adjusted to pH 7.4 with sodium hydroxide. In all studies the fat cells were incubated in

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The abbreviation used is: cyclic AMP, cyclic adenosine 3', 5'-monophosphate.
**Effect of propranolol and dihydroergotamine on lipolysis and cyclic AMP accumulation in white fat cells**

White fat cells (12 mg per tube) were incubated for 1 hour in 1.5 ml of medium containing 4% albumin. The basal values are the means of seven experiments, and the increments due to the inhibitors as the mean ± standard error of the paired differences. All agents were added at the start of the incubation period. The zero time value for labeled cyclic AMP accumulation was 114 cpm per mg.

| Additions                   | Cyclic 3',5'-AMP | Glycerol release |
|-----------------------------|------------------|------------------|
| None                        | 67               | 1                |
| Epinephrine, $5 \times 10^{-7}$ M | 123              | -48 ± 48         |
| Epinephrine, $1.3 \times 10^{-6}$ M | 157              | -96 ± 48         |

In all studies the fat cells were isolated and incubated at 37°C.

Unpublished results.
Fig. 2. Stimulation by growth hormone (GH) and dexamethasone (DEX) of lipolysis and cyclic AMP accumulation after a lag period of 1 hour. White fat cells (approximately 33 mg per tube) were incubated for periods ranging from 1 to 4 hours in the presence of 2 mM theophylline. The values are the means of four paired experiments, and significant effects of 0.75 pg of growth hormone per ml and 0.016 pg of dexamethasone per ml are indicated by an asterisk (p < 0.05 by paired comparisons). Glycerol release is shown as micromoles per g of cells and cyclic AMP accumulation as counts per min per mg of cells. The zero time value for labeled cyclic AMP accumulation was 12 cpm per mg.

in a shaking water bath with air as the gas phase. All values for lipolysis and cyclic AMP accumulation in each experiment were based on the average of duplicate tubes and are the changes over the incubation period based on initial controls which were incubated for 5 min.

Phosphorylase was determined by the following modification of the method of Diamond and Brody (18). After the incubation period the fat cells from six replicate tubes for each point were pooled, separated by centrifugation from the albumin medium, suspended in 2 ml of cold (0-5°) 50 mM Tris buffer (pH 6.8) containing 1 mM EDTA, 20 mM NaF, and 0.3% albumin and homogenized with a Potter-Elvehjem homogenizer (Teflon pestle). After centrifugation at 26,000 x g for 10 min at 0°, 0.2-ml aliquots of the infranatant (the soluble supernatant below the fat cake and above the sedimented material) were incubated in 50 mM Tris buffer (pH 6.8), 0.4% glyceogin, 10 mM glucose 1-phosphate, 1 mM EDTA, 20 mM NaF, and 0.3% albumin in a total volume of 1 ml. The rate of production of inorganic phosphate was constant for 1 hour. No phosphorylase activity was detectable if glycogen was omitted from the incubation medium. In all experiments phosphorylase activity was also measured in the presence of 1 mM AMP, which only resulted in an increase in all tubes of phosphorylase activity by 5 pmoles of phosphate per g of cells. The values for phosphorylase in each experiment were based on the average of duplicate tubes. The significance of the differences due to growth hormone in the phosphorylase experiments was determined by Student's t test (19) on the logarithms of the paired differences. Logarithmic transformation was done to normalize the distribution of the data (19).

Bovine growth hormone (NIH GH-B6) was a gift of the Endocrinology Study Section of the National Institutes of Health, and dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione), of Merck and Company. L-Norepinephrine, L-epinephrine, theophylline, propranolol, dactinomycin, puromycin, and cycloheximide were obtained commercially. Adenine-αH (7 Ci per mmole) was obtained from New England Nuclear, and adenine-8-3H (22 Ci per mmole) and adenine-8-14C (50 mCi per mmole) were obtained from Schwarz BioResearch.

RESULTS

Catecholamines have been shown to increase total cyclic AMP accumulation in white adipose tissue (4) and cells (20). We found that epinephrine also increased both cyclic AMP accumulation and lipolysis in the absence of theophylline (Fig. 1). The addition of 1 mM theophylline markedly increased lipolysis, and no further increase in lipolysis was seen in the presence of epinephrine. The addition of theophylline (1 mM) alone did not
increase the accumulation of labeled cyclic AMP but did potentiate the stimulation by epinephrine of cyclic AMP (Fig. 1). Similar results have been reported by Kuo and De Renzo (21), who measured the total cyclic AMP accumulation. The latter authors found no effect of 1-land (20), who measured the total cyclic AMP accumulation. Similar results have been reported by Kuo and De Renzo (21), who used the same system, and by Butcher, Baird, and Sutherland (20).

Table I indicates that the increase in cyclic AMP accumulation due to epinephrine was blocked by propranolol, a specific β-adrenergic antagonist (22). Propranolol also blocked the lipolytic action of epinephrine (Table I). Dihydroergotamine, which is a specific antagonist of the lipolytic action of catecholamines (23), inhibited the lipolytic action of the lower concentration of epinephrine used in Table I. Dihydroergotamine alone increased cyclic AMP accumulation in the absence but not in the presence of catecholamines and 1 mM theophylline or caffeine (20).

The addition of growth hormone and dexamethasone (a synthetic glucocorticoid) to fat cells increased cyclic AMP accumulation and lipolysis after a lag period of 1 hour (Fig. 2). At 3 hours both lipolysis and cyclic AMP accumulation were significantly greater than in controls (Fig. 2). The increment in glycerol release due to the addition of growth hormone and glucocorticoid was low since a high concentration of theophylline (2 mM) was present in all incubation flasks. At lower concentrations of theophylline (0.2 or 0.5 mM) the effect of growth hormone and dexamethasone on lipolysis was greater than in the presence of 2 mM theophylline (Fig. 2). These results are similar to those previously reported from this laboratory where growth hormone and dexamethasone increased the sensitivity of fat cells to low concentrations of theophylline but not the maximum lipolytic response (10). Growth hormone and dexamethasone in the absence of theophylline increased the accumulation of cyclic AMP (Fig. 3). In three of the seven experiments shown in Fig. 3, cyclic AMP levels and glycerol release were also measured at 1 hour. Growth hormone and dexamethasone did not increase either cyclic AMP accumulation or glycerol release at 1 hour at any concentration of theophylline. The results from the medium and the cyclic 3', 5'-AMP content of each were determined separately. The control values shown are the means of six paired experiments, and the increments due to the hormones are the mean ± standard error of the paired differences. The zero time values for labeled cyclic AMP accumulation in cells was 100 and in the medium, 75 cpm per mg.

**Table I**

| Additions         | Cyclic 3', 5'-AMP  | Cyclic AMP accumulation in cells |
|-------------------|--------------------|----------------------------------|
|                   | cpm/mg             |                                  |
| None              | 86                 |                                  |
| Puromycin         | 84                 |                                  |
| Theophylline      | +13 ± 5            | +4 ± 8                           |
| Theophylline + cycloheximide | +10 ± 0          | 0 ± 3                            |
| None              | 154                | 206                              |
| Theophylline      | +3 ± 10            | +64 ± 8                          |
| Theophylline + cycloheximide | -1 ± 11          | -3 ± 6                           |
| Free fatty acid accumulation in cells
|                   | pmoles/g           |                                  |
| None              | -1                 | 9                                |
| Theophylline      | +6 ± 2             | +9 ± 4                           |
| Theophylline + cycloheximide | 0 ± 1            | 0 ± 2                            |
| Free fatty acid accumulation in medium
|                   | pmoles/g           |                                  |
| None              | 2                  | 19                               |
| Theophylline      | +10 ± 5            | +23 ± 4                          |
| Theophylline + cycloheximide | -2 ± 2           | +2 ± 6                           |
| Glycerol release to medium
|                   | pmoles/g           |                                  |
| None              | 2                  | -1                              |
| Theophylline      | +15 ± 1.5          | +15 ± 2                          |
| Theophylline + cycloheximide | -1 ± 2           | +4 ± 3                           |
**Table IV**

**Effect of epinephrine, growth hormone, and dexamethasone on accumulation of cyclic 3',5'-AMP in medium**

White fat cells (19 mg per tube) were incubated for 4 hours in 1.5 ml of medium containing 4% albumin, 0.1 mg per ml of ascorbate, 0.4 mM theophylline, and 0.1 mg per ml of ovomucoid. Growth hormone (0.075 μg per ml), dexamethasone (0.016 μg per ml), and epinephrine (1.3 × 10^{-5} M) were added at the start of the incubation. At the end of the incubation period as much of the medium as possible was separated from the cells and analyzed separately for accumulation of labeled and free fatty acids. The values shown are the means of four paired experiments, and significant effects of added agents are indicated by an asterisk (p < 0.05 by paired comparisons). The zero time values for labeled cyclic AMP accumulation in cells was 25, and in the medium, 22 cpm per mg.

| Additions            | Accumulation of 3',5'-AMP | Accumulation of free fatty acids |
|----------------------|---------------------------|---------------------------------|
|                      | Cells Medium | Cells Medium |                      |                          |
|                      | cpm/mg      | μmoles/g     |                      |                          |
| None                 | 72 70        | 23 66        |                      |                          |
| Dexamethasone        | 65 81        | 27 74        |                      |                          |
| Growth hormone       | 86 92        | 27 83*       |                      |                          |
| Growth hormone +     | 64 144*      | 32* 91*      |                      |                          |
| Epinephrine          | 100 354*     | 40* 87*      |                      |                          |

* to be a dissociation between the increase in glycerol release due to theophylline alone and the accumulation of cyclic AMP. As the glycerol release increased with higher concentrations of theophylline in the presence or absence of growth hormone and dexamethasone, cyclic AMP accumulation was reduced (Fig. 3).

The action of growth hormone and dexamethasone on the stimulation of lipolysis has been hypothesized to require protein synthesis (8, 9). Puromycin (10^{-4} M), an inhibitor of protein synthesis, inhibited the increment in the accumulation of cyclic AMP due to growth hormone and dexamethasone, as well as the increment in glycerol release (Table II).

Cycloheximide, an antibiotic which blocks protein synthesis at a different site from puromycin, blocked the increased free fatty acid and glycerol release due to growth hormone and dexamethasone (Table III). The effects of this antibiotic on cyclic AMP accumulation were more complicated. Cycloheximide increased cyclic AMP accumulation in both the cells and the medium at 2 and 4 hours in both the presence and the absence of growth hormone and dexamethasone (Table III). There was no added increase in cyclic AMP accumulation due to growth hormone and dexamethasone in the presence of cycloheximide (Table III).

Growth hormone and dexamethasone increased the intracellular accumulation at the end of 2 hours of incubation, but at 4 hours nearly all the labeled cyclic AMP was in the medium (Table III). The increase in glycerol release due to growth hormone and dexamethasone was significantly enhanced in the cells at the end of 2 and 4 hours (Table III).

The possibility that the increased accumulation of labeled cyclic AMP in the medium but not in the cells was unique to growth hormone and glucocorticoid action was excluded by the studies shown in Table IV. At the end of 4 hours of incubation, epinephrine also markedly increased the accumulation of cyclic AMP in the medium without having any significant effect on that accumulated in the cells.

The combination of both glucocorticoid and growth hormone was required to increase the accumulation of labeled cyclic AMP (Table IV). Neither hormone alone significantly stimulated free fatty acid or cyclic AMP accumulation except for growth hormone alone with regard to free fatty acid release to the medium (Table IV).

If the accumulation of labeled cyclic AMP represents an increase in the total cyclic AMP content of cells, then one would also expect to see an increase in the phosphorylase activity of fat cells due to growth hormone since this enzyme is activated by cyclic AMP (1-3). It has been shown that catecholamines activate both lipolysis and phosphorylase in white adipose tissue (3). The results in Fig. 4 indicate that the activity of phos-
phorylase in homogenates of rat fat cells was enhanced by prior incubation of the cells for \(3\frac{1}{2}\) hours with growth hormone and dexamethasone. Cycloheximide blocked the increase in phosphorylase activity due to growth hormone but had no effect on basal activity, which was largely due to the addition of 1 mM theophylline 30 min before the end of the fat cell incubation. The effect of growth hormone on phosphorylase activity correlated well with that on lipolysis in the same experiments (Fig. 4).

**Discussion**

Fain, Galton, and Kovacev (24) found that theophylline potentiated the lipolytic action of growth hormone and dexamethasone. Their experiments suggested that, if theophylline is solely acting by inhibiting the activity of the cyclic AMP phosphodiesterase, the lipolytic response of fat cells to growth hormone and dexamethasone might be mediated by cyclic AMP. The increased release of labeled cyclic AMP to the medium by fat cells incubated in the presence of growth hormone and glucocorticoid shown in the present studies provides more direct evidence for an effect of these hormones on cyclic AMP metabolism.

If intracellular cyclic AMP formation is increased by growth hormone and glucocorticoid, then one would also expect to see an increase in the activity of glycogen phosphorylase. Not only was there an increase in glycogen phosphorylase seen in the presence of these hormones, but the action of growth hormone and glucocorticoid was blocked by cycloheximide (Fig. 4). Eisen and Goodman (25) have also reported an increase in glycogen phosphorylase activity of homogenates prepared after incubation of intact adipose tissue with growth hormone and glucocorticoid.

In our experiments we have assumed that the amount of labeled cyclic AMP released to the medium is a reflection of the physiologically active intracellular pool of cyclic AMP. One would also like to make the further assumption that an increased release of cyclic AMP is due to increased intracellular formation rather than to decreased degradation. Although we found similar effects of growth hormone in the absence as well as in the presence of theophylline, this does not prove that the increase in intracellular cyclic AMP was due to increased formation.

A problem in equating radioactivity in the cyclic AMP fraction with its rate of formation is the requirement that the specific radioactivity of the ATP which serves as the precursor of cyclic AMP not be altered by the various lipolytic and antilipolytic agents. This is difficult to exclude, and we must consider the possibility that growth hormone and glucocorticoid may alter the specific activity of the ATP pool. Experiments determining the total amount of ATP are, however, only of relative value, if we assume that the adenine nucleotides within the cell exist in intracellular pools (26). If there is a small pool of ATP specific for adenyl cyclase activity, this pool may fluctuate totally independently of all other intracellular ATP. Until methods are available for locating and determining specific ATP pools, this problem will remain unsolved. More conclusive proof of a direct interaction of growth hormone and adenyl cyclase awaits the indication that adenyl cyclase activity of cells is increased in the presence of growth hormone and glucocorticoid. However, our results indicate that the addition of growth hormone and dexamethasone to fat cells does increase the accumulation of labeled cyclic AMP and the activation of phosphorylase, which supports the hypothesis that the total cyclic AMP accumulation is increased by these hormones.

Epinephrine markedly increased the accumulation of labeled cyclic AMP in the presence or absence of theophylline. However, a concentration of theophylline (1 mM) which resulted in the maximal stimulation of lipolysis had no effect on labeled cyclic AMP content. Butcher et al. (20) have also reported that 10 mM caffeine had little effect on the absolute amount of cyclic AMP in fat cells. The finding that increasing the concentration of theophylline in the medium did not increase the accumulation of labeled cyclic AMP but actually decreased it indicates that little of the newly made cyclic AMP is being degraded by a phosphodiesterase sensitive to the concentrations of theophylline used in our experiments. Only in the presence of concentrations of theophylline which already maximally stimulated lipolysis was there further accumulation of cyclic AMP when lipolytic agents were added. These results suggest that there may be multiple pools of cyclic AMP or theophylline may have multiple effects.

Most of the accumulation of labeled cyclic AMP at the end of 4 hours of incubation was in the medium. Whether this transfer process is physiological or an artifact of the fat cell preparation procedure is not immediately known. Ashman et al. (27) have reported that cyclic AMP synthesized within body cells transverses “cell walls” to the plasma and then penetrates the glomerular membrane. Schönhöfer and Skidmore (4) found that 40% of the newly made cyclic AMP produced in fat cells as a result of the addition of epinephrine leaves the cells within 10 min. Kuo and De Renzo (21) reported similar results except over a time span of 60 min.

The slow onset and the inhibition by puromycin of both cyclic AMP accumulation and lipolysis support the hypothesis that the action of growth hormone and glucocorticoid is to increase the synthesis of a protein or proteins which results in increases in the cyclic AMP pool of white fat cells.

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