Effects of Insulin on the Levels of Adenosine 3':5'-Monophosphate and Lipolysis in Isolated Rat Epididymal Fat Cells*

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SUMMARY

Effects of insulin on the level of adenosine 3':5'-monophosphate (cyclic AMP) and the rate of lipolysis in isolated rat epididymal fat cells were determined under various conditions. When cells were incubated with 0.01 to 100 nM insulin, it gave: (a) a biphasic inhibitory effect that was maximal between 0.1 and 1 nM and submaximal at either lower or higher insulin concentrations on lipolysis stimulated by 0.5 mM dibutyryl cyclic AMP, 10 nM adrenocorticotrophic hormone (ACTH), or 1 to 10 μM norepinephrine; (b) a monophasic inhibitory effect that was maximal at 1 to 100 nM on lipolysis induced by 5 or 20 mM cyclic AMP or 1 to 4 mM caffeine; and (c) a monophasic stimulatory effect that was maximal at 10 to 100 nM on lipolysis induced by 1 mM dibutyryl cyclic AMP or 0.1 to 1 mM norepinephrine.

Under the above conditions, lipolysis was either inhibited or stimulated by either 10 times recrystallized Novo insulin or a highly purified single component insulin, but not by modified insulin preparations that were inactive in stimulating glucose utilization in fat cells. On the other hand, neither the inhibitory nor stimulatory effect of insulin was observed when the cellular insulin receptor or receptors were modified by exposure of cells to trypsin (1 mg per ml) for 15 min. Insulin at either 0.2 or 10 nM significantly decreased the cellular level of cyclic AMP in the presence or absence of various lipolytic agents tested, except in the presence of high concentrations (0.1 to 1 mM) of catecholamines (see below). The effect of insulin to lower the cellular level of cyclic AMP was seemingly large enough to account for the antilipolytic effect of this hormone when the cyclic AMP level was low (or when the determinations were made in the presence of 2 to 5 mM caffeine, 10 to 100 mM glucagon, or less than 10 μM ACTH) but not when the nucleotide level was high (or when the determinations were made in the presence of 1 μM norepinephrine or 10 to 100 nM ACTH).

Insulin at 10 nM further increased the levels of cyclic AMP and lipolysis that were highly elevated by 0.1 to 1 mM norepinephrine, whereas insulin at 0.2 nM had no significant effects on the two parameters under these conditions. On the other hand, either in the absence of any lipolytic agent or in the presence of 2 mM caffeine or 10 nM ACTH, insulin at 10 nM lowered the level of cyclic AMP to a larger degree than it did at 0.2 nM. Consequently, the effect of insulin on the cyclic AMP level was monophasic while its effect on lipolysis was biphasic (see above) in the presence of 10 nM ACTH.

The effects of the insulin like action of trypsin on the levels of cyclic AMP and lipolysis were similar to those of insulin at 0.2 nM. Incidentally, all the effects of norepinephrine noted above were observed with epinephrine.

The above mentioned insulin effects do not necessarily indicate the physiological effects of this hormone since they were observed in the presence of wide concentration ranges of insulin and certain lipolytic agents. Thus, the above data suggest that, under the given in vitro conditions, insulin (rather than its possible contaminants) induces multiple effects on the levels of cyclic AMP and lipolysis depending on its concentration and on the nature and concentrations of the lipolytic agent used. Part of the difference in the effects of lipolytic agents might be explained by the difference in the cyclic AMP levels elevated by these agents. Since all the insulin effects tested seem to be mediated by the cellular insulin receptor or receptors, it is suggested that the insulin receptor system of fat cells can respond to a wide concentration range (approximately from 0.01 to 100 nM) of insulin.

As it is well known, insulin inhibits lipolysis in adipose tissue cells under certain conditions (1). According to the hypothesis proposed by Butcher et al. (2, 3) one of the mechanisms by which insulin induces this effect is to lower the cellular level of cyclic AMP. The basis of this hypothesis was the observation that insulin lowered the cellular cyclic AMP concentration when it was greatly elevated by a joint action of epinephrine and caffeine (2, 3). It should be noted, however, that insulin did not inhibit lipolysis under these conditions (i.e. in the presence of both epinephrine and caffeine) and that it was technically difficult to detect any insulin effects on the nucleotide level when it was slightly elevated with either epinephrine or caffeine alone (3). Subsequently, it was reported by Sneyd et al. (4) and Manganiello et al. (5) that insulin at approximately 7 (4) and 70 nM (5) did significantly lower the cyclic AMP level that was raised by

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epinephrine alone. However, in apparent disagreement with these observations, it was recently noted by Fain and Rosenberg (6), Jarett et al. (7), and Kono and Barham (8) that insulin, especially at low physiological concentrations, did not detectably decrease the cellular level of cyclic AMP under certain conditions in which the antilipolytic effect of the hormone was seen. In addition, it was recently reported from several laboratories that, under selected conditions, insulin gave a biphasic (8–10) or a stimulatory effect (10, 11) on lipolysis.

Accordingly, the present work was initiated to reinvestigate the effects of insulin on the cellular level of cyclic AMP and the rate of lipolysis under various conditions. The specific aim of the work presented in this paper are to test the working hypotheses that: (a) insulin itself (rather than its possible contaminants) induces both inhibitory and stimulatory effects on lipolysis; (b) that it does so as a result of interaction with the cellular insulin receptor or receptors; and (c) the effects of insulin on lipolysis are not necessarily secondary to its effects on the cellular level of cyclic AMP. Preliminary observations of this work have been published (12, 13).

MATERIALS AND METHODS

Crude bovine serum albumin (Fraction V) was purchased from Schwarz-Mann (Lot No. W 4255); N\textsubscript{6},O\textsubscript{4′}-dibutyryl cyclic AMP, from Boehringer Mannheim; cyclic AMP, catecholamines, and theophylline, from Sigma; ACTH and some epinephrine preparations, from Parke-Davis; and caffeine, from Merck. Glucagon and the single-component insulin (14) were gifts from the Eli Lilly Company. Ten time recrystallized insulin was a gift from Dr. Schlichtkrull of Novo Laboratories; this insulin (Lot No. 0-27667; 24.4 units per mg) was routinely used throughout this study. Modified insulin preparations that were inactive in stimulating the conversion of glucose to CO\textsubscript{2} were prepared by exposing insulin to (a) 0.1 N NaOH for 10 min at 90°, (b) trypsin (1 mg per ml) for 15 min at 37°, or (c) ethylmercaptol (15). After the inactivation, NaOH was neutralized with HCl, trypsin was inactivated with soybean trypsin inhibitor, and ethylmercaptol was removed (15). The concentrations of hormones and other lipolytic agents are shown in molarities, which were estimated on a weight basis on the assumption that the materials were 100% pure, except in the case of ACTH. The concentration of the latter was estimated from the indicated biological activity on the assumption that the activity of pure ACTH is 200 units per mg (16). Labeled cyclic [\textsuperscript{3H}]AMP (16.3 Ci per mmole) was purchased from Schwarz-Mann. The cyclic AMP-binding protein and the inhibitor protein were prepared in this laboratory as described by Gilman (17).

The isolated fat cells were prepared by the collagenase method (18) from the epididymal adipose tissue of Sprague-Dawley rats (150 to 200 g). The buffer used for the incubation of fat cells was Krebs-Ringer bicarbonate supplemented with crude bovine serum albumin (20 mg per ml), pH 7.4, at 37°. The incubation of fat cells and subsequent analysis were carried out under two slightly different conditions. In the first part of the study where only the rate of lipolysis was determined without measuring the level of cyclic AMP, freshly prepared fat cells, approximately 5 mg, were incubated in a total volume of 1 ml. The cells were first placed in a water bath shaker at 37° for 10 to 20 min (see below) prior to use. Insulin, when present, was added at this stage. Lipolysis was induced by the addition of a lipolytic agent and the incubation was continued for 30 min. No glucose was added to the incubation mixture. Catecholamine was freshly dissolved in 0.1 M HCl containing 154 mM NaCl; addition of this solution to 9 volumes of the incubation mixture did not change the pH of the buffer. Lipolysis was always started at 90 to 100 min after the adipose tissue was removed from the animals since it was noted that both the sensitivity and the maximal response of fat cells to a lipolytic agent gradually increased with time. For the determination of fatty acid accumulation, lipolysis was terminated by the addition of 1 N H\textsubscript{2}SO\textsubscript{4} (0.2 ml). Fatty acid was assayed by the titrimetric method of Dole and Meinertz (19). For the assay of glycerol, lipolysis was terminated by the addition of 4.6 M perchloric acid (0.1 ml). The mixture was supplemented with 0.1 ml of chloroform, mixed, and centrifuged. An aliquot of the aqueous phase was adjusted to pH 4 to 6 with KOH. Glycerol was assayed by the enzymatic method of Wieland (20).

In the second part of the study where the levels of both cyclic AMP and lipolysis were determined, 70 to 90 mg of fat cells were incubated in a total volume of 3 ml. Lipolysis was initiated by adding either a methylxanthine or a lipolytic hormone after freshly prepared cells were kept at 37° for 10 to 20 min, or until 90 to 100 min after the tissue was removed from the animals. The incubation was continued for 15 min unless stated otherwise. At the end of the incubation, the reaction mixture was supplemented with 0.2 ml of 6.8 M perchloric acid, 0.1 ml of a tracer quantity of cyclic [\textsuperscript{3H}]AMP, and 0.4 ml of chloroform. The entire mixture was homogenized by a brief sonication and centrifuged. An aliquot (2 ml) of the supernatant solution was applied to a column (3.5 x 55 mm) of AG 50 x 8 (200 to 400 mesh, from Bio-Rad that had been equilibrated with 50 mM HCl; the column was subsequently washed with 1 mM acetic acid). Glycerol was recovered almost quantitatively in the first 2.9 ml of the effluent, and the majority of cyclic AMP was fractionated into the next 1.4 ml (within 2 hours after the sample was applied to the column). The glycerol fraction eluted from the column was neutralized with 1 N KOH; glycerol was assayed by the enzymatic method of Wieland (20). The cyclic AMP fraction (in 1 mM acetic acid) was supplemented with 0.1 ml of NaOH-acetate buffer of an appropriate concentration to make the final acetate concentration 50 mM and the pH 4.0. The recovery of cyclic AMP was estimated from the recovery of the added cyclic [\textsuperscript{3H}]AMP. The amount of cyclic AMP was determined by the binding-protein method of Gilman (17) with a few modifications, and the results were calculated using the equation of Hales and Randle (21). When the extracellular level of cyclic AMP was to be determined, the incubation mixture was supplemented with a tracer quantity of cyclic [\textsuperscript{3H}]AMP at the end of the incubation and centrifuged for 1 min. An aliquot (2.5 ml) of the aqueous layer was withdrawn, mixed with 0.2 ml of perchloric acid, and then treated under the standard conditions as described above. Since metabolic activities of adipose tissue are considerably

\textsuperscript{3}T. Kono, unpublished observation.

\textsuperscript{4}The final concentration of cyclic [\textsuperscript{3H}]AMP was reduced to 3 to 4 nM. This increased the sensitivity of the assay. Although cyclic [\textsuperscript{3H}]AMP did not saturate the binding protein at this concentration range, a linear standard curve was obtained by processing the data with the equation of Hales and Randle (21). The time for incubation of cyclic AMP with the binding protein was increased to overnight (at 0–5°). After the reaction mixture was diluted with the pH 6 buffer (17), three aliquots of the same solution were filtered with different pieces of Millipore filter and processed separately. This enabled us to detect and eliminate falsely low data that were occasionally obtained as a result of an incomplete binding of the label to the filter membrane. The mean value of the three (or two) data obtained by these procedures was counted as one observation (n = 1) in statistical analyses.
different from animal to animal (22, 23), data to be compared were obtained using several aliquots (usually three for each point) of a pooled cell suspension. All the observations were then confirmed by repeating the experiments on at least one other occasion using another batch of fat cells. The validity of these procedures has been discussed (24, 25). The amount of fat cells in a preparation was estimated from the content of malate dehydrogenase (8).

RESULTS

Multiplicity of Insulin Effects on Lipolysis and Requirement of Hormone Receptors

Effects of Insulin on Lipolysis Induced by Cyclic AMP or Dibutyryl Cyclic AMP—The data in Table I indicate that insulin (a) gave no detectable effect on the basal level of lipolysis in isolated fat cells at any hormone concentration tested, (b) greatly inhibited lipolysis induced by 5 or 20 \text{nM} cyclic AMP when the hormone concentration was \text{0.1 nM} or above, and (c) affected the reaction induced by dibutyryl cyclic AMP in a complex manner. Thus, when the concentrations of dibutyryl cyclic AMP were 0.5, 1, and 3 \text{mM}, insulin had a biphasic, a stimulatory, and no detectable effect, respectively. These data in Table I bring together the isolated observations previously reported from different laboratories that insulin inhibited lipolysis induced by the added cyclic AMP (10, 11, 26) and showed a biphasic (10), a stimulatory (10, 11), or no detectable effect (27, 28) on the reaction induced by dibutyryl cyclic AMP. However, the present data do not confirm the previous reports that insulin had a biphasic (10) or no significant effect (28) on lipolysis induced by the added cyclic AMP.

The stimulatory effect of insulin observed above in the presence of 1 \text{mM} dibutyryl cyclic AMP was characterized further in the experiments summarized in Table II. The data obtained with intact cells (not treated with trypsin) indicate that the stimulatory effect was observed with either Novo insulin or a highly purified single-component insulin, but not with modified hormone preparations (Table II, Footnote a). These observations suggested that the stimulatory effect was induced by insulin itself, rather than by a possible contaminant. In addition, the data in the last two columns in Table II indicate that the stimulatory effect of insulin, but not that of dibutyryl cyclic AMP, was blocked by trypsin treatment of fat cells. This treatment is known to modify the cellular receptors for insulin and certain other peptide hormones without significantly affecting the other elements in fat cells (29).

Effects of Insulin on Lipolysis Induced by Methylxanthines—As is well known, insulin inhibits lipolysis induced by caffeine. When the concentration of caffeine was increased from 1 to 4 \text{mM}, both the apparent sensitivity (\(K_C = 0.1 \text{ to } 1.2 \text{ nM}\)) and the maximal response of the system to insulin were decreased (Fig. 1). This suggested that the effects of caffeine and insulin were not entirely "competitive," in disagreement with the data reported by Hepp et al. (28). Although the \(K_C\) values noted above are significantly larger than those observed in the presence of lipolytic hormones (see below), the inhibitory effect of insulin on lipolysis induced by caffeine was completely blocked by trypsin treatment of fat cells (1 mg per ml for 15 min; data not shown), as was the insulin effect on lipolysis induced by epinephrine (8).

Effects of Insulin on Lipolysis Induced by Lipolytic Hormones—As noted previously (8, 9), the effect of insulin to inhibit lipolysis induced by 10 \text{nM ACTH} or 1 to 10 \text{\mu M} norepinephrine was bi-

### TABLE I

<table>
<thead>
<tr>
<th>Additon</th>
<th>Untreated cells</th>
<th>Trypsin-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 5 \text{mM} cyclic AMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 20 \text{mM} cyclic AMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.5 \text{mM} DBCA</td>
<td></td>
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</tr>
<tr>
<td>+ 1 \text{mM} DBCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 3 \text{mM} DBCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Lipolysis: glycerol accumulation**

- 11.1 ± 1.1\(^a\) 37.7 ± 1.3 86.6 ± 3.0 57.5 ± 2.3 224 ± 6 627 ± 10
- 9.8 ± 1.2 17.7 ± 0.5 21.6 ± 0.7 34.6 ± 0.2 205 ± 18 622 ± 9
- 10.2 ± 1.1 11.0 ± 0.5 13.3 ± 0.7 39.7 ± 2.7 285 ± 9 619 ± 21
- 11.7 ± 1.6 11.4 ± 1.5 13.9 ± 0.7 41.8 ± 1.5 301 ± 12 633 ± 3
- 11.1 ± 0.9 10.0 ± 1.9 15.3 ± 0.6 53.1 ± 1.6 322 ± 8 618 ± 16

**nMoles/glycerol/30 min/10 mg cells**

- The values obtained with insulin modified by NaOH treatment, trypsin treatment, and reduction were 94 ± 7, 93 ± 5, and 88 ± 5 (\(n = 3\)), respectively.

**a** n.d., not determined.

**Footnote a** Basal activity.

**Footnote b** Mean value ± S.E. (\(n = 3\)); this is applicable to all the tables in this paper.
peaks of lipolytic activity at concentrations of 1 and 300 pM.

Finally noted by Hllcn et al. (30), norepinephrine alone gave two concentration ranges of norepinephrine were studied (Fig. 3). As originally noted by Allen et al. (30), norepinephrine alone gave two peaks of lipolytic activity at concentrations of 1 and 300 µM. The present data show that insulin at 0.1 nM greatly lowered the first peak without significantly affecting the second peak, whereas the hormone at 10 nM partially lowered the first peak and greatly elevated the second peak. The effects of insulin at 1 nM were in-between the two types of effects mentioned above. The dual effect of insulin (i.e., the inhibition of the first peak and stimulation of the second peak) could also be observed (a) when norepinephrine was replaced with epinephrine or isoproterenol or (b) when lipolysis was measured by the accumulation of fatty acid instead of glycerol (data not shown). In contrast, neither the double peak of lipolysis nor the dual effect of insulin was observed when 1 to 1000 nM ACTH was used in place of catecholamine (data not shown).

The dual effect of Novo insulin observed in the presence of 1 and 100 µM norepinephrine could be reproduced with the highly purified single-component insulin (Table III). In contrast, all of the modified insulin preparations tested failed to stimulate lipolysis induced by 100 µM norepinephrine (Table III). In addition, 0.1 µM to 10 µM glucagon (a contaminant of some insulin preparations) added to the incubation mixture failed to increase the rate of lipolysis that was stimulated by 100 µM epinephrine or norepinephrine (data not shown). It appeared, therefore, that high concentrations of insulin (rather than its possible contaminants) stimulated lipolysis under the given conditions. This stimulatory effect was half-maximal when the insulin concentration was approximately 0.6 nM (Fig. 4). In addition, it is shown in Fig. 4 that the stimulatory effect of insulin, but not that of norepinephrine alone, was completely blocked when fat cells were exposed to trypsin (1 mg per ml) for 15 min. This observation is consistent with the view that this stimulatory effect, as well as other insulin effects noted earlier, was induced as a result of interaction of the hormone with its cellular receptor.

### Effects of Insulin on the Levels of Cyclic AMP and Lipolysis

#### Effects of Insulin Observed at Different Incubation Periods—
The data in Figs. 5 and 6 show the insulin effects observed on

### TABLE III

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glycerol accumulation (nmol/30 min/10 mg cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20 ± 4</td>
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<tr>
<td>1 µM Norepinephrine</td>
<td>241 ± 8</td>
</tr>
<tr>
<td>+ Novo insulin</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>+ Single-component insulin</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>100 µM Norepinephrine</td>
<td>187 ± 3</td>
</tr>
<tr>
<td>+ Novo insulin</td>
<td>338 ± 4</td>
</tr>
<tr>
<td>+ Single-component insulin</td>
<td>341 ± 2</td>
</tr>
<tr>
<td>+ NaOH-treated &quot;insulin&quot;</td>
<td>168 ± 2</td>
</tr>
<tr>
<td>+ Trypsin-treated &quot;insulin&quot;</td>
<td>162 ± 2</td>
</tr>
<tr>
<td>+ Reduced &quot;insulin&quot;</td>
<td>188 ± 8</td>
</tr>
</tbody>
</table>

![Fig. 1](image1.jpg)

![Fig. 2](image2.jpg)

![Fig. 3](image3.jpg)

![Fig. 4](image4.jpg)

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Fig. 1 (left). Effects of insulin concentration on lipolysis induced by several levels of caffeine. Fat cells were incubated with caffeine at a level of 1 (○), 2 (●), 3 (△), or 4 mM (□) in the presence of insulin at the concentrations shown in the abscissa. Each point in Figs. 1 through 4 represents the mean value of two parallel experiments.

Fig. 2 (right). Effects of insulin on lipolysis induced by norepinephrine, ACTH, or glucagon. Fat cells were incubated with insulin at the concentrations shown in the abscissa. Each point in Figs. 1 through 4 represents the mean value of two parallel experiments.

Fig. 3 (left). Effects of insulin on lipolysis induced by a wide concentration range of norepinephrine. Fat cells were incubated with the indicated concentrations of norepinephrine plus insulin at a level of 0 (○), 0.1 (●), 1.0 (●), or 10 nM (△).

Fig. 4 (right). Effects of insulin on lipolysis induced by 100 µM norepinephrine in untreated and trypsin-treated fat cells. Untreated (control) fat cells (○) and cells treated with trypsin (●; 1 mg per ml for 15 min) were incubated with 100 µM norepinephrine plus insulin at the indicated concentrations.
by Manganiello et al. (5), the peak of the nucleotide observed in the present study, a peak of cyclic AMP was observed for convenience at 15 min in the rest of the experiments presented in this paper. Incidentally, the levels of insulin used in these experiments (either 0.2 or 10 nm) were those that would give the maximal (or close to the maximal) effects on lipolysis under selected conditions (see Figs. 1 through 4). It is of interest to note that, according to the data shown above, the levels of the nucleotide and lipolysis were not shown). The extracellular level of cyclic AMP calculated from the data in Table IV was 24.6 ± 3.5% of the total; similar results were obtained under other conditions tested, at 15 min of incubation. Accordingly, it was assumed in the present study that the changes in the total amount of cyclic AMP could be used as an indicator of the changes in the cellular level of the nucleotide.

The rest of the data in Table IV (Project C) indicate that insulin significantly decreased the basal as well as the ACTH-dependent levels of cyclic AMP, although the effect of the hormone to inhibit the basal rate of lipolysis was undetectably small (see also Table I).

Effects of Insulin Concentration—As shown in Fig. 9, the level of cyclic AMP was decreased to a greater extent by insulin at 10 nm than at 0.2 nm either in the absence of any lipolytic agent (Panel A) or in the presence of 2 mM caffeine (Panel B) or 10 nM ACTH (Panel C). However, such an effect of insulin was not as obvious in the presence of 1 mM norepinephrine (Panel D). It is possible that under these conditions (Panel D), the lowering effect of 10 nM insulin was partially counteracted by the stimulatory effect of the hormone that was predominantly observed in the presence of higher concentrations of norepinephrine (see Figs. 6 and 8). On the other hand, effects of insulin on lipolysis were: (a) undetectably small in the absence of any lipolytic agent, (b) monophasically inhibitory in the presence of 2 mM caffeine, and (c) biphasically inhibitory in the presence of 10 nM ACTH or 1 mM norepinephrine (Fig. 9). The mono- and biphasic effects of insulin in the presence of ACTH on the levels of cyclic

**Fig. 5 (left).** Effects of insulin observed at different times of incubation experiments with 1 mM norepinephrine and 0.2 nm insulin. In the experiments shown in this and the next figure (Fig. 6), fat cells were incubated with the indicated concentrations of norepinephrine and insulin for the periods shown in the figures. Each column and small bar shows the mean value of three observations and the standard error of the mean. White column, no hormones; dotted column, + 1 mM norepinephrine; and shaded column, + 1 mM norepinephrine + 0.2 nM insulin. The levels of cyclic AMP shown in this and other figures in this paper are the total amounts of the nucleotide in the incubation mixture (per g of cells). Of the total amount of cyclic AMP, approximately 25% was extracellular under various conditions tested, at 15 min of incubation (see Fig. 4). The rest of the data in Table IV (Project C) indicate that insulin significantly decreased the basal as well as the ACTH-dependent levels of cyclic AMP, although the effect of the hormone to inhibit the basal rate of lipolysis was undetectably small (see also Table I).

Effects of Insulin on Two Parameters Increased by Different Lipolytic Agents—As shown in Figs. 7 and 8, the levels of cyclic AMP and lipolysis were lowered to similar extents by insulin when the two parameters were increased by either 2 to 5 mM caffeine or 10 to 100 nM glucagon (Fig. 7) but not when they were elevated by either 10 to 100 nM ACTH or 1 to 1000 mM norepinephrine (Fig. 8). Thus, in the presence of 10 to 100 nM ACTH, 0.2 nm insulin moderately lowered the cyclic AMP level and largely inhibited lipolysis (Fig. 8). A similar unparallel effect of insulin was observed in the presence of 1 mM norepinephrine (Fig. 8; see also Figs. 5 and 9). Furthermore, it is shown in Fig. 8 that in the presence of 0.1 to 1 mM norepinephrine, 10 nM insulin increased the levels of both cyclic AMP and lipolysis (see also Fig. 6) and that under the same conditions, 0.2 nM insulin had no significant effects on the two parameters. Incidentally, the levels of insulin used in these experiments (either 0.2 or 10 nm) were those that would give the maximal (or close to the maximal) effects on lipolysis under selected conditions (see Figs. 1 through 4). It is of interest to note that, according to the data shown above, the levels of the nucleotide and lipolysis were not shown). The extracellular level of cyclic AMP calculated from the data in Table IV was 24.6 ± 3.5% of the total; similar results were obtained under other conditions tested, at 15 min of incubation. Accordingly, it was assumed in the present study that the changes in the total amount of cyclic AMP could be used as an indicator of the changes in the cellular level of the nucleotide.
AMP and lipolysis were confirmed by experiments carried out with a wide concentration range of insulin (Table V).

**Insulin-like Effects of Trypsin—**Trypsin, in its insulin-like action (8), significantly lowered the concentration of cyclic AMP either in the absence or in the presence of 10 nM ACTH (Table VI). The enzyme also inhibited lipolysis induced by 10 nM ACTH. These effects of the enzyme were smaller than, but similar to, the corresponding effects of insulin at 0.2 nM. In addition, in this particular set of experiments, both trypsin and insulin seemed to have slightly lowered the basal level of lipolysis although this effect of insulin was usually undetectable (Tables I and IV). Incidentally, the effects of trypsin observed on the cyclic AMP level were too large to be accounted for by any non-specific proteolytic destruction of fat cells, as judged from our previous data (8).

**Relationship between Effects of Insulin on Levels of Cyclic AMP and Lipolysis—**When the levels of glycerol accumulated by fat cells in the presence of 0 to 5 mM caffeine were plotted against the levels of cyclic AMP in the same samples, a hypothetical dose-response curve was obtained relating the levels of the two parameters (Fig. 10). Significantly, the points representing the data obtained in the presence of 0 to 5 mM caffeine plus 10 nM insulin (0.2 nM in two cases) were also found, when plotted on the same graph, in the vicinity of this hypothetical curve (Fig. 10). This observation is consistent with the view that the effect of insulin to inhibit lipolysis stimulated by 0 to 5 mM caffeine may largely be accounted for by the hormonal effect to decrease the cyclic AMP level (see also Figs. 7 and 9). By contrast, when fat cells were incubated with either 0 to 100 nM ACTH or 0 to 100 nM ACTH plus 0.2 nM insulin, two separate apparent dose-response curves were obtained (Fig. 11). This result is in agreement with the earlier observation that the effect of 0.2 nM insulin to lower the cyclic AMP level elevated by 10 to 100 nM ACTH was considerably smaller than its antilipolytic effect observed under the same conditions (see Figs. 8 and 9). It should be noted, however, that the lower portions of the two curves in Fig. 11 are indistinguishable at the region where the levels of cyclic AMP are less than approximately 0.3 n mole per g of cells. This observation can be interpreted to indicate that, even in the presence of ACTH, the effect of insulin on lipolysis may largely be accounted for by its effect on the cyclic AMP level when the latter level is sufficiently low and is less than 0.3 n mole per g in the presence of insulin. In this regard, it is of interest to note that the maximal level of cyclic AMP recorded in Fig. 10 in the presence of caffeine plus insulin was also approximately 0.3 n mole per g.

In addition, it is seen in Fig. 11 that the data obtained with cells that had been exposed to the "insulin-like" action of trypsin were in the vicinity of the lower (plus-insulin) curve, whereas those obtained in the presence of 10 nM insulin were not, presumably because the hormone gave mono- and biphasic effects on the levels of the two parameters (see Fig. 9). Incidentally, the lower portions of the curves shown in Figs. 10 and 11 are probably concave and almost horizontal at the basal level of cyclic AMP.
TABLE IV
Effects of insulin on total, extracellular, or basal level of cyclic AMP and glycerol

Fat cells were incubated for 15 min under standard conditions either in the presence or absence of 0.2 nm insulin and of the indicated concentrations of lipolytic hormones. In Projects A and B, the total and extracellular levels of cyclic AMP and glycerol were determined. In Project C, only the total levels of the two parameters were determined after the cells were incubated for 15 min in the presence or absence of 10 nm ACTH as shown. The data listed under different experimental numbers were obtained with different batches of fat cells. The figures in the table show the mean value and S.E. (n = 3).

<table>
<thead>
<tr>
<th>Project, experimental number, and conditions</th>
<th>Cyclic AMP</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ Insulin</td>
</tr>
<tr>
<td></td>
<td>µmoles/g cells</td>
<td>µmoles/g cells</td>
</tr>
<tr>
<td>(1) Total</td>
<td>0.554 ± 0.023</td>
<td>0.434 ± 0.005c</td>
</tr>
<tr>
<td>(1) Extracellular</td>
<td>0.145 ± 0.009</td>
<td>0.153 ± 0.003c</td>
</tr>
<tr>
<td>(2) Total</td>
<td>0.442 ± 0.026</td>
<td>0.323 ± 0.042c</td>
</tr>
<tr>
<td>(2) Extracellular</td>
<td>0.109 ± 0.009</td>
<td>0.091 ± 0.011c</td>
</tr>
<tr>
<td>(3) Total</td>
<td>1.206 ± 0.024</td>
<td>0.900 ± 0.035c</td>
</tr>
<tr>
<td>(3) Extracellular</td>
<td>0.357 ± 0.184</td>
<td>0.125 ± 0.013c</td>
</tr>
<tr>
<td>(4) Total</td>
<td>1.076 ± 0.020</td>
<td>0.915 ± 0.001c</td>
</tr>
<tr>
<td>(4) Extracellular</td>
<td>0.151 ± 0.021</td>
<td>0.119 ± 0.003c</td>
</tr>
</tbody>
</table>

- a When compared to the controls by Student’s t test, p < 0.01.
- b When compared to the controls by Student’s t test, p > 0.10.
- c When compared to the controls by Student’s t test, p < 0.10.
- d When compared to the controls by Student’s t test, p < 0.05.

TABLE V
Effects of insulin concentration on the levels of cyclic AMP and glycerol

Fat cells were incubated with 10 nm ACTH plus the indicated concentrations of insulin.

<table>
<thead>
<tr>
<th>Insulin concentration</th>
<th>Cyclic AMP</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/g cells</td>
<td>µmoles/g cells</td>
</tr>
<tr>
<td>0</td>
<td>0.346 ± 0.017</td>
<td>4.01 ± 0.08</td>
</tr>
<tr>
<td>1</td>
<td>0.264 ± 0.001</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.267 ± 0.018</td>
<td>1.52 ± 0.03</td>
</tr>
<tr>
<td>100</td>
<td>0.260 ± 0.017</td>
<td>3.07 ± 0.06</td>
</tr>
</tbody>
</table>

since it was shown in Table IV and Fig. 9 that insulin gave minimal and significant effects on the basal levels of lipolysis and cyclic AMP, respectively. Results similar to those shown in Fig. 11 were obtained when cells were incubated with 0 to 1 µM of catecholamine or 0 to 1 µM of catecholamine plus 0.2 nm insulin (data not shown).

DISCUSSION

In the experiments shown above, cells were incubated with wide concentration ranges of insulin and certain lipolytic agents. Consequently, the experimental results do not necessarily indicate the physiological effects of insulin in vivo. Nevertheless, the experimental data are of interest from the mechanistic standpoint for the analysis of insulin action at the molecular level. Of the multiple effects of insulin on lipolysis, the inhibitory effects are generally observed with physiological concentrations (less than approximately 1 nm (32)), while the stimulatory effects are typically seen with unphysiologically high concentrations (approximately 1 to 100 nm) of the hormone. Consequently, it might appear that the stimulatory effects are induced by possible contaminants in the hormone preparation or by unknown pharmacological actions of insulin that may not be mediated by its cellular receptor. However, the experiments with highly purified and modified insulin preparations indicate that both types of effects are probably induced by insulin itself. In addition, the data obtained with cells exposed to trypsin (1 mg per ml for 15 min) indicate that both inhibitory and stimulatory actions of insulin are presumably mediated by cellular hormone receptors that are modifiable with the enzyme. In this regard, trypsin is known to modify not only the insulin receptor but also the glucagon receptor (29) and part of the ACTH receptor (33); however, it is highly unlikely that insulin stimulates lipolysis upon interaction with the receptors for these lipolytic hormones since insulin alone does not stimulate lipolysis even at high hormone concentrations. Besides, the stimulation of lipolysis by insulin is observed in the presence of certain lipolytic hormones rather than methylxanthines. It appears, therefore, that stimulation, as well as inhibition, of lipolysis by insulin is mediated by the cellular insulin receptor or receptors. Consequently, it is suggested that the insulin receptor system of fat cells can respond to a wide concentration range (approximately from 0.01 to 100 nm) of the hormone. This suggestion is consistent with
Fat cells were exposed to trypsin (1 mg per ml) for 15 s and the enzyme was subsequently inactivated with soybean trypsin inhibitor (8). The trypsin-treated cells were incubated under standard conditions either in the absence or presence of 10 nM ACTH as shown. As controls, intact cells were incubated under the same conditions either in the absence (Control 1) or presence of 0.2 nM insulin (Control 2). The data listed under different experimental numbers were obtained with different batches of fat cells.

The dissociation constants (Kd values) estimated for the binding of insulin to the majority of fat cell receptors were between 3 and 7 nM (34-37). It is conceivable, therefore, that the stimulatory effects are induced by a specific or an extremely strong hormonal signal that is thought to be generated by these high Kd receptors in response to insulin at unphysiologically high concentrations. A possible physiological role of these high Kd receptors has been discussed (34, 35, 38).

Contrary to several recent observations (6-8), the present data indicate that insulin at either 0.2 or 10 nM concentration significantly decreases the cyclic AMP level under various conditions except in the presence of high concentrations (0.1 to 1 mM) of catecholamine. Furthermore, present data obtained in the presence of less than 5 mM caffeine (Figs. 7 and 10), 10 to 100 nM glucagon (Fig. 7), or less than 10 nM ACTH (Fig. 11, at the region where the two apparent dose-response curves are indistinguishable) are consistent with the view that, when the level of cyclic AMP is elevated only moderately, the effect of insulin to inhibit lipolysis may largely be accounted for by the hormonal effect to lower the nucleotide level. On the other hand, it is also shown in the present work that when the level of cyclic AMP is highly elevated by 1 mM norepinephrine or 10 to 100 nM ACTH, the relative effect of 0.2 nM insulin to lower the nucleotide level is apparently too small to explain the antilipolytic effect of this hormone (Table IV and Figs. 5, 8, 9, and 11). In fact, insulin gives such a small relative effect on the nucleotide level under these conditions that it was undetectable in our previous study (8). It should be noted, however, that the absolute effect of insulin on the nucleotide level stayed about the same, or slightly lower the nucleotide level. On the other side, it was previously argued that unless cyclic AMP (e.g. Ref. 39). However, this argument seems to be in disagreement with the current concept of the kinase action (40). Al-

According to the current concept (40), protein kinase (BC, B = regulatory subunit and C = catalytic subunit) is activated by cyclic AMP (A) by the following reaction:

\[ A + BC = AB + C \]
ternatively, the above observation that insulin inhibits lipolysis to a larger degree than it depresses the cyclic AMP level can be interpreted to indicate that insulin inhibits lipolysis by an unknown mechanism instead of, or in addition to, that which is mediated by a decrease in the concentration of cyclic AMP. The presence of such mechanisms has been considered previously in connection with the studies of insulin actions in muscle (41) and liver (42). In addition, it is probably safe to assume that there is a cyclic AMP-independent mechanism of insulin action in fat cells since the effects of insulin on glucose transport (1) and pyruvate dehydrogenase (43) in fat cells have never been correlated with the hormonal effect on the cyclic AMP level. Incidentally, the role of cyclic AMP in the insulin effect on protein synthesis is controversial (7, 44).

The effects of insulin at 10 nM observed in the present study are very complex. According to the data, the hormone at this concentration either decreases or increases the levels of cyclic AMP and lipolysis depending on the nature and concentration of the lipolytic agent used. The difference in the effects of the lipolytic agents might reflect the difference in their unknown characteristics or the levels of cyclic AMP elevated by these lipolytic agents. Since insulin alone increases neither the level of cyclic AMP nor that of lipolysis (Fig. 9), the stimulation of the two parameters by insulin in the presence of 0.1 to 1 mM of norepinephrine (Figs. 6 and 8) may involve an unknown synergistic reaction between high concentrations of insulin and norepinephrine. Similarly, the second phase of a biphasic antilipolytic effect of insulin (Table 1 and Figs. 2 and 9) may also involve a synergism between high concentrations of insulin and selected concentrations of cyclic AMP or dibutyryl cyclic AMP. Since insulin gives monophasic and biphasic effects on the levels of cyclic AMP and lipolysis that are elevated by 1 mM catecholamine or 10 nM ACTH (Fig. 9 and Table V), it is suggested that some of the previous data obtained with high concentrations of insulin (4, 5, 25) should be interpreted with caution. On the other hand, since insulin gives only monophasic effects on the two parameters in the presence of caffeine (Fig. 9), large and clear-cut effects of insulin can be observed on the levels of cyclic AMP and lipolysis when 10 nM insulin is used in experiments with this lipolytic agent (Figs. 7, 9, and 10).

As a possible mechanism of insulin action, it was recently reported by Hepp (45) and Lilliano and Cuatrecasas (46) that insulin gave a biphasic inhibitory effect on adenylate cyclase in broken cell systems. Their observations are seemingly incompatible with the present data which indicate that the effect of insulin on protein synthesis is controversial (7, 44).

The equilibrium constant \( K \) of this reaction is:

\[ K = \frac{[A][BC]}{[AB][C]} \]

In this equation, \([BC] = [C] \) when the activation is half-maximal; hence, the cyclic AMP concentration \( K_a \) that causes the half-maximal stimulation is:

\[ K_a = [A]_0 = K\cdot[AB] \]

or, if \([B] = [C] \),

\[ K_a = K\cdot \frac{1}{2} \text{ [total } BC] \]

This indicates that the \( K_a \) value in the cell should be higher than the \( K_a \) value observed in a cell-free system, in which the kinase concentration is relatively low.

**Addendum**—During the preparation of this paper, it was reported by Khoo et al. (52) that a physiological concentration of insulin lowered the level of cyclic AMP raised by epinephrine to a significant extent, but not at all in proportion to the reduction in lipolysis. Their data seem to be consistent with those obtained in the present study under selected conditions.

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Effects of Insulin on the Levels of Adenosine 3':5'-Monophosphate and Lipolysis in Isolated Rat Epididymal Fat Cells
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