Effects of Lipolytic and Antilipolytic Agents on Cyclic 3',5'-Adenosine Monophosphate in Fat Cells

VINCENT C. MANGANELLO, FERID MURAD* AND MARTHA VAUGHAN

From the Molecular Disease Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Isolated fat cells were incubated in Krebs-Ringer phosphate medium. Within 1 min after the addition of 1.1 mM epinephrine, the cyclic AMP content of the cells was elevated. It continued to rise, reaching a peak in 4 to 7 min, and fell thereafter, being only slightly above the control levels after 10 to 15 min. A maximal rate of glycerol production was established within 1 to 2 min, well before the peak in cyclic AMP concentration, and was maintained unchanged while the cyclic AMP level continued to rise and then fell. Both in magnitude and time course, the effect of adrenocorticotropin (ACTH), 0.2 unit per ml, on cyclic AMP levels and on lipolysis was exactly like that of epinephrine (or of both hormones together). Addition of more epinephrine or of ACTH at several times during 1 hour of incubation with 1.1 mM epinephrine did not change cyclic AMP levels. The effects of epinephrine on cyclic AMP levels and on glycerol production were unaltered by the presence in the medium of 4 mM glucose.

When propranolol (or insulin) was added several times after epinephrine-simulated lipolysis had begun, the rate of glycerol production fell to zero within 1 to 2 min. In the same period, the cyclic AMP concentration fell to a basal level; i.e. the rate of decrease was much greater than that observed after 4 to 7 min of incubation with epinephrine alone. Furthermore, after incubation of cells for 20 min with epinephrine, the addition of 0.8 mM theophylline produced an 8-fold rise in cyclic AMP content measured 5 min later. This increment was, however, only a fraction of that observed during the first 5 min after addition of epinephrine in the absence of theophylline. Since propranolol inhibits the action of epinephrine but does not alter basal adenyl cyclase activity, and, since 0.8 mM theophylline alone did not alter cyclic AMP levels, it may be concluded that adenyl cyclase was still epinephrine-stimulated during the time when cyclic AMP levels were falling. Whether the rate of formation of cyclic AMP during this period was equal to that observed with the initial addition of epinephrine. Cyclic AMP levels, however, rose little if at all in 4 to 5 min after addition of ACTH. The ability of cells to elevate cyclic AMP levels a second time in response to hormonal stimulation was restored when cells that had been incubated for 20 min were washed and suspended in fresh medium. There appears to be something accumulated in the medium during incubation of cells with epinephrine that interferes with the effectiveness of the hormone in causing accumulation of cyclic AMP in fresh cells. Whether this material has its effect on the rate of formation or of degradation of cyclic AMP cannot be decided.

Butcher et al. (1, 2) first studied the effects of several lipolytic and antilipolytic substances on cyclic AMP levels in fat cells incubated with caffeine to inhibit cyclic nucleotide phosphodiesterase. They pointed out that "measurements of lipolysis in these experiments were of little value, for . . . cyclic AMP levels were far in excess of those required for maximal rates of lipolysis." More recently, Kuo and De Renzo (3) investigated the effects of several hormones and other agents on accumulation of radioactive cyclic AMP by fat cells previously incubated with adenine-\(^{3}C\) to label adenine nucleotide pools. Since no effect of ACTH or of norepinephrine was observed in the absence of phosphodiesterase inhibition, theophylline was included in the medium. Fatty acid production was measured in these experiments as an index of lipolysis, although in most experiments fatty acid production, which was already elevated because of the presence of the methyl xanthine, was not further increased by ACTH or epinephrine. In contrast to the findings of Rodbell (4), and for reasons that are not clear, stimulation of fatty acid production was not observed until 15 to 20 min after addition of hormone or theophylline (or both), whereas cyclic AMP levels were elevated in less than 5 min. Although cyclic AMP levels were elevated before fatty acid production was enhanced in these experiments, as the authors pointed out, lipolysis could be stimulated (to what

* Present address, Department of Pharmacology and Medicine, University of Virginia, Charlottesville, Virginia 22901.
was apparently a maximal level with theophylline alone) with little or no change in "C-cyclic AMP. We undertook to determine cyclic AMP levels and rates of glycerol production (as an index of lipolysis) in the absence of methyl xanthines during the periods of onset and cessation of hormone action.

In the experiments of Kuo and De Renzo (3), accumulation of "C-cyclic AMP continued for 20 to 30 min in the presence of norepinephrine and theophylline, after which the level fell. Butcher et al. (1) had found that in the presence of hormone and theophylline the peak level of cyclic AMP was reached somewhat earlier, at about 6 min, but also in their studies the level declined thereafter. It seemed possible that this decrease might be due to a diminution of methyl xanthine inhibition of phosphodiesterase with time. In the presence of hormone alone, however, we observed a similar phenomenon and have attempted to learn whether the decline in cyclic AMP levels that occurs despite the continued presence of hormone is secondary to a decrease in the rate of cyclic AMP formation or an increase in the rate of its destruction (or both). Some of these observations have been reported in abstract form.  

**METHODS AND MATERIALS**

Fat cells were prepared from epididymal fat pads of Osborne-Mendel rats (180 to 175 g) and incubated as previously described (5). Fat cell weight was estimated from the amount of hexane-extractable, hydroxamate-reactive ester (6) assuming 3 µeq of ester to be equivalent to 1 mg of cells. When glycerol production and cyclic AMP levels were measured, 150 to 250 mg of cells were incubated in a total volume of 3 ml of Krebs-Ringer phosphate medium containing 30 µg per ml of bovine serum albumin in polyethylene vials at 37° with gentle shaking. In experiments in which glycerol production only was measured, each incubation vial contained 30 to 50 mg of cells in a total volume of 2.5 ml. After incubation for 30 min, hormones and other agents were added. All incubations were done in duplicate. At several times during incubation, samples of cells plus medium were taken for determination of glycerol, and the remainder (cells plus medium) was mixed with 20 ml of 0.02 M HCl containing 10,000 cpm (4 pmoles) of "H-cyclic AMP, essentially as previously described (7).

Samples were heated (100°) for 10 min, filtered, and neutralized with KOH, and Tris-HCl buffer (pH 7.4) was added to a final concentration of 0.02 M. Samples were treated twice with 2 ml each of 0.32 M ZnSO₄ and 0.3 M Ba(OH)₂. The supernatants were applied to small columns (0.5 x 2.5 cm) of Dowex 2 previously washed with 0.02 M Tris, pH 7.4. The columns were then washed with an additional 15 ml of the same buffer, and cyclic AMP was eluted with 4 ml of 0.1 M HCl. The eluates were lyophilized, taken up in 0.5 ml of 0.02 M Tris, pH 7.4, and applied to small columns (0.5 x 3.0 cm) of Dowex 50. The columns were washed with 1.5 ml of H₂O. The next 3 ml were collected, taken to dryness, and dissolved in 0.2 ml of 0.02 M Tris, pH 7.4. Purified samples were assayed for cyclic AMP content with a liver phosphodiesterase activation assay system (8). Recoveries of "H-cyclic AMP (30 to 45%) were used to calculate the quantities of cyclic AMP in unpurified extracts.

In experiments in which cyclic AMP distribution (cells versus medium) was determined, the contents of three or four incubations were pooled and a sample (cells plus medium) was removed and processed as above. The remainder was centrifuged at 600 x g for 1 min; a sample of medium was aspirated from beneath the floating fat cells and processed as above. Glycerol production (micromoles per g of cells) is reported as the mean of values from duplicate incubations corrected for the nonincubated controls. In most instances, glycerol production in control incubations was less than 2 to 5 µmoles per g per hour. These basal values have been omitted in several figures. Cyclic AMP levels (nanomoles per g of cells) are reported for each incubation except where noted.

"H-Cyclic AMP (4.86 Ci per mmole) was purchased from New England Nuclear. Amorphous insulin was a gift from Dr. O. K. Behrens of Eli Lilly. Other hormones and agents were obtained as previously described (7).

**RESULTS**

*Changes in Cyclic AMP Content and Lipolysis at Onset and Cessation of Hormonal Stimulation*—In 23 experiments, the cyclic AMP content at the beginning of the incubation period was 0.5 ± 0.08 n mole of cyclic AMP per g (mean ± S.E.). The amount of cyclic AMP in the system (cells plus medium) was in most experiments essentially constant for as long as 2 hours of incubation (7). Under these conditions, the rate of glycerol production was usually less than 2 to 4 µmoles per g per hour.

As shown in Table I, 0.6 to 1.4 µg epinephrine produced an apparently maximal elevation of cyclic AMP levels determined 4 min after addition of the hormone. A similar level was reached after addition of ACTH, 1.25 units per ml. The maximal levels observed 4 min after addition of glucagon were always somewhat lower. The same maximal rates of lipolysis were produced by 0.55 to 1.1 µg epinephrine or 0.2 to 0.5 unit per ml of ACTH, whereas the maximal rate attainable with glucagon was lower (Table II). Combinations of two or three of these hormones (in maximally effective concentrations) produced rates of glycerol production no greater than those achieved with epinephrine or ACTH alone. In all of the experiments reported here, epinephrine and ACTH were used in concentrations that produced maxi-
Table II

Effect of hormones individually and in combination on glycerol production

Glycerol content of cells plus medium was determined at 0, 10, 20, and 30 min after addition of hormones. In all incubations the rate of glycerol production was constant throughout this period. Data presented are the means of 30-min values in duplicate vials.

| Hormone added                  | Concentration | Glycerol produced (nmol/g/30 min) |
|--------------------------------|---------------|----------------------------------|
| Glucagon                       | 0.5 μg/ml     | 42.1                             |
|                                | 1.0 μg/ml     | 41.2                             |
|                                | 0.55 μM       | 54.0                             |
|                                | 1.1 μM        | 53.7                             |
| Epinephrine                    | 0.2 unit/ml   | 52.4                             |
|                                | 0.5 unit/ml   | 53.8                             |
| ACTH                           | 1.1 μM        | 55.4                             |
| Epinephrine plus ACTH          | 0.5 unit/ml   | 55.4                             |
| Epinephrine plus glucagon      | 1.1 μM        | 56.6                             |
| ACTH plus glucagon             | 1.0 μg/ml     | 55.2                             |
| Epinephrine plus glucagon      | 0.5 unit/ml   | 55.2                             |
| Epinephrine plus ACTH          | 1.1 μM        | 55.4                             |
| Epinephrine plus glucagon      | 0.5 unit/ml   | 55.0                             |

Fig. 1. Effects of epinephrine on cyclic AMP content and glycerol production in fat cells plus medium. Cells were incubated for 30 min before addition of 1.1 μM epinephrine. Each point represents cyclic AMP content or glycerol production in a single incubation.

Fig. 2. Effects of epinephrine on glycerol production and cyclic AMP accumulation and distribution between cells and medium. Cells were incubated for 30 min. Epinephrine, 1.1 μM, was then added and, at the indicated times, samples of cells plus medium and of medium free of cells were taken for determination of glycerol and cyclic AMP content. Each point represents the mean of values from duplicate incubations. The difference between total cyclic AMP content of cells plus medium and the cyclic AMP content of the medium was taken to represent intracellular cyclic AMP. The concentration of cyclic AMP in cell water was calculated assuming that intracellular water equals 5% of cell weight.

Table II shows the effect of hormones individually and in combination on glycerol production. As shown in Fig. 1, within 1 min after addition of 1.1 μM epinephrine, the cyclic AMP level was elevated. It was still higher at 4 min, but, despite the continued presence of epinephrine, fell thereafter and was only slightly above the control at 10 min. A maximal rate of glycerol production, on the other hand, was established within 1 to 2 min, well before the peak in cyclic AMP concentration, and was maintained unchanged while the cyclic AMP level continued to rise and then fell. Both in magnitude and time course, the effect of ACTH on cyclic AMP levels and on lipolysis was exactly like that of epinephrine (Figs. 6 and 10). After addition of either hormone, the level of cyclic AMP reached a peak in 4 to 7 min and declined thereafter.

It is important to note that, whereas most of the cyclic AMP that accumulates during the first few minutes following the addition of epinephrine is intracellular (Fig. 2), we have previously found (7) that after incubation for more than 30 min under a variety of conditions the amount of cyclic AMP present in the medium exceeds the amount present in the cells. Thus, in the experiments reported below in which total cyclic AMP content of cells plus medium was determined, changes during the first 10 min were followed. When the amount of cells incubated was less than 25 to 30 mg per ml, a constant apparently maximal rate of lipolysis was observed for more than 30 min. In most experiments in which cyclic AMP was measured, however, larger amounts of cells were used, and the initial rate of glycerol production was maintained for only 10 to 15 min, after which it decreased, presumably due to the accumulation of free fatty acids (7).
Cells were incubated with or without 4 min after addition of hormones can be assumed to represent changes in intracellular cyclic AMP, but this may not be true at later times. (Butcher et al. (1) also reported that most of the cyclic AMP accumulated during 10 min of incubation with epinephrine and caffeine was intracellular.)

Stimulation of glycerol release by epinephrine can be prevented by the prior or simultaneous addition of insulin or propranolol, and these compounds prevented the rise in cyclic AMP concentration when fat cells were incubated with caffeine plus epinephrine (1). As shown in Table III, insulin and propranolol prevented the effect of epinephrine alone on cyclic AMP concentration in fat cells, but did not alter basal levels. The effect of epinephrine on glycerol production in these experiments was also inhibited. When insulin (Fig. 3) or propranolol (Fig. 4) was added at several times after epinephrine-stimulated lipolysis had begun, the rate of glycerol production fell essentially to zero within 1 to 2 min. In the same period of time, as shown in Fig. 5, the cyclic AMP concentration fell to a basal level. In these experiments in which propranolol was added 4 min after epinephrine (or ACTH), the β-adrenergic blocking agent had no effect on cyclic AMP levels in control incubations or in those with ACTH. It had no effect on glycerol production except in the incubations which contained epinephrine. In those, glycerol production ceased within 1 min after addition of propranolol (data not shown). When epinephrine-stimulated lipolysis had ceased following addition of propranolol, the lipolytic system was still capable of responding maximally to ACTH (Fig. 6).

Factors Related to Decline in Cyclic AMP Concentration after 4 to 7 Min of Incubation with Hormone—It seems clear that the initial rapid rise in cyclic AMP concentration that follows addition of epinephrine (or ACTH) is the result of hormonal stimulation of cyclic AMP formation, the rate of which then exceeds the rate of its destruction. After 4 to 7 min, however, despite the continued presence of the hormone, cyclic AMP levels fall; i.e., the rate of cyclic AMP formation is less than the rate of destruction. It is apparent that cyclic AMP synthesis is still stimulated, since the addition of propranolol, which does not alter basal adenyl cyclase activity but inhibits the effect of epinephrine, caused the cyclic AMP content to drop much more rapidly than it did in the presence of epinephrine alone (or ACTH alone, or ACTH plus propranolol) (Fig. 5). If the decline in cyclic AMP

### Table III

| Addition, concentration     | Cyclic AMP (nmol/g) |
|-----------------------------|---------------------|
| None                        | 0.75 (0.5, 1.0)     |
| Insulin, 0.2 milliunits/ml  | 0.80 (0.8, 0.8)     |
| Propranolol, 5 μM           | 0.85 (0.8, 0.9)     |
| Epinephrine, 1.1 μM         | 1.70 (1.6, 1.8)     |
| Epinephrine + propranolol   | 0.80 (0.8, 0.8)     |
| Epinephrine + insulin       | 1.00 (1.0, 1.0)     |

**Fig. 3** (left). Effect of insulin on glycerol production. Cells were incubated with or without 0.6 milliunit per ml of insulin for 30 min. Epinephrine, 0.27 μM, was then added to cells previously incubated with or without insulin. At several times after the addition of epinephrine, insulin, 0.6 milliunit per ml, was added (D) to some incubations. The mean and range of duplicate incubations are presented. Glycerol production in controls and in the presence of insulin alone was the same as in cells incubated with insulin prior to the addition of epinephrine (data not shown).

**Fig. 4** (middle). Effect of propranolol on glycerol production. Cells were incubated with or without 4 μM propranolol for 30 min. Epinephrine, 0.08 μM, was then added to cells previously incubated with or without propranolol. At several times after the addition of epinephrine, 4 μM propranolol was added to some incubations. Data are presented as in Fig. 3. Glycerol production in controls and in the presence of propranolol alone was the same as in cells incubated with propranolol prior to the addition of epinephrine. Propranolol did not inhibit the lipolytic effects of 50 milliunit per ml of ACTH (data not shown).

**Fig. 5** (right). Effect of propranolol on cyclic AMP content of fat cells previously incubated with epinephrine or ACTH. After incubation for 30 min, 1.1 μM epinephrine (○) or 0.04 unit per ml of ACTH (□) was added in Experiment A. In Experiment B only epinephrine (●) was added. Four minutes later 5.0 μM propranolol was added to incubations already exposed to epinephrine (○) or ACTH (□). O, no additions. Each point represents the mean cyclic AMP content (cells plus medium) of duplicate incubations. The cyclic AMP content after incubation for 7 min with propranolol alone was 1.6 nmoles per g in Experiment A, and 0.4 nmoles per g in Experiment B. In these experiments, propranolol completely blocked the lipolytic effect of epinephrine but did not affect either control values or glycerol production stimulated by ACTH.
levels observed within 4 to 7 min after hormone addition were solely related to the cessation of hormonal stimulation of cyclic AMP formation, cyclic AMP levels should fall as precipitously as observed when propranolol is added to epinephrine-treated cells. Furthermore, after incubation of cells for 20 min with epinephrine, the addition of 0.8 mM theophylline (which in the absence of epinephrine did not alter cyclic AMP levels) produced an 8-fold rise in cyclic AMP content measured 5 min later (Fig. 7). This increment was, however, only a fraction of that observed during the first 5 min after addition of epinephrine in the presence of theophylline. Such a difference would result if the rate of cyclic AMP formation (although stimulated relative to control incubations) were in fact less after 20 to 25 min of incubation with epinephrine than it was at 0 to 5 min. Alternatively, it could be due to changes in cyclic AMP degradation during the incubation with epinephrine.

If there is in fact a decline in the rate of cyclic AMP formation, it is probably not due to a decrease in the effective concentration of hormone, since addition of ACTH after 7.5 min of incubation with epinephrine (Fig. 8) or of more epinephrine after 10 min (Fig. 9A) did not prevent the further decline of cyclic AMP levels during the next 4 min. In the experiment shown in Fig. 10, after 25 min of incubation with epinephrine or ACTH, addition of the same or combinations of different hormones produced no significant elevation of cyclic AMP at 30 min. After 56 min (Fig. 9B) of incubation with epinephrine, a second addition of the same hormone had no effect on cyclic AMP levels measured 4 min later, although addition of epinephrine to control flasks at 56 min induced a 170% increase in cyclic AMP content.

A decrease in the rate of cyclic AMP formation could also occur despite maximal stimulation of adenylyl cyclase activity if the concentration of substrate (ATP) became limiting. There is reason to believe that in isolated fat cells (3) and in brain slices (9, 10) cyclic AMP is formed from a pool of ATP that does not readily equilibrate with the total intracellular ATP. Although, under the conditions of our experiments, changes in total fat cell ATP would be expected to be minimal (11), even small changes, if confined primarily to a cyclic AMP precursor pool, might well be significant. It has been reported that addition of glucose to the medium largely prevents the decrease in ATP content that otherwise occurs when fat cells from fasted rats are incubated with epinephrine (11). As shown in Fig. 11, however, glucose had no effect on cyclic AMP content (or on glycerol production) during incubation for 18 min with epinephrine, nor did the presence of glucose alter the failure of cells incubated with epinephrine for 20 min to respond to a second addition of epinephrine (Fig. 7).

Whatever changes take place in the cell that lead to the decline in cyclic AMP levels despite the continued presence of a maximally effective concentration of hormone, it is evident that they occur very quickly. Not only do levels begin to decrease between 4 and 7 min after hormone addition, but the rate of accumulation of cyclic AMP is clearly more rapid in the first 1 to 2 min than it is later (Figs. 1 and 12). These changes also appear to persist after the effect of epinephrine has been terminated. In several experiments, propranolol was added 4 min after epinephrine. At various times from 7 to 20 min later ACTH was added. In most experiments cyclic AMP levels rose little if at all in 4 to 5 min after addition of ACTH, although glycerol production which had ceased after addition of propranolol resumed immediately at a rate equal to that observed with the initial addition of epinephrine (Fig. 6). In the experiment presented in
Fig. 8 (left). Effect of hormone readdition on cyclic AMP content of fat cells plus medium. Cells were incubated for 30 min before addition of 1.4 μM epinephrine or 0.54 unit per ml of ACTH. After 7.5 min ACTH was added to cells previously exposed to epinephrine, and the incubations continued for 4 min. Data are presented as in Fig. 7.

Fig. 9 (middle). Effect of epinephrine readdition on cyclic AMP levels. Cells were incubated for 30 min before addition of 1.4 μM epinephrine. In Experiment A a second addition of 1.4 μM epinephrine was made 15 min later. In Experiment B, 1.4 μM epinephrine was added 55 min later both to control cells and to cells already exposed to 1.4 μM epinephrine. Each point represents the mean values from duplicate incubations.

Fig. 10 (right). Effect of hormone readdition on cyclic AMP content of fat cells plus medium. Cells were incubated for 30 min before addition of 1.1 μM epinephrine or 0.5 unit per ml of ACTH. After 25 min additional epinephrine (1.1 μM) or a combination of 0.5 unit per ml of ACTH and 1.2 μg per ml of glucagon was added to cells incubated with epinephrine. To cells incubated with ACTH was added additional ACTH or a combination of 1.1 μM epinephrine and 1.2 μg per ml of glucagon. Incubations were then continued for 5 min. Data are presented as in Fig. 9.

Fig. 11. Effect of glucose on cyclic AMP content of cells plus medium. Cells were incubated in the presence or absence of 4 mM glucose for 30 min before 1.4 μM epinephrine was added. Data are presented as in Fig. 9.

Fig. 12, the addition of propranolol 4 min after epinephrine did not completely stop lipolysis, and 7 min later the cyclic AMP was still elevated above the control level. Addition of ACTH at this time increased glycerol production to the initial maximal rate, and 4 min later the cyclic AMP level was almost double that in incubations with epinephrine and propranolol to which no ACTH has been added. The increment in cyclic AMP which was the largest observed in experiments of this type was nevertheless considerably smaller than that observed when ACTH was added to cells that had been incubated under control conditions for the same period.

The ability of the cells to elevate cyclic AMP levels a second time in response to hormonal stimulation was restored when cells that had been incubated for 20 min with epinephrine were washed and suspended in fresh medium (Experiment 1, Table IV). In Experiment 2, Table IV, medium separated from cells after incubation for 20 min with epinephrine caused a 150% increase in the cyclic AMP content of fresh cells incubated in it for 4 min. A similar increase was observed in fresh cells incubated with this medium plus additional epinephrine. On the
Effects of epinephrine on cyclic AMP levels in previously incubated cells and on fresh cells in “used” medium

In Experiment 1, cells were incubated for 20 min with or without 1.1 μm epinephrine (after an initial 30-min incubation period in medium alone). They were then separated by flotation as described under “Methods and Materials,” washed once with medium (27°), suspended in fresh medium, and incubated for 4 min with or without epinephrine. In Experiment 2, cells were incubated for 20 min with or without 1.1 μm epinephrine. Cells were separated from the medium and discarded. Samples of both media were taken for determination of cyclic AMP content. Incubations were set up with 2 ml of either control or epinephrine “used” medium with or without added epinephrine, 1.1 μm. Fresh fat cells were added and incubated for 4 min before determination of cyclic AMP content of cells plus medium. Cyclic AMP contributed by the “used” medium has been subtracted from these values. Data are reported as in Table I.

Table IV

| Experiment and cells | Medium | Epinephrine, 11 μμ | Cyclic AMP (nmole/g) |
|----------------------|--------|-------------------|---------------------|
| 1. Incubated without epinephrine | Fresh | 0 | 0.30 (0.3, 0.3) |
| Incubated with epinephrine | Fresh | 0 | 0.25 (0.2, 0.3) |
|                       | Fresh | + | 0.50 (0.5, 0.5) |
| 2. Fresh              | Control | 0 | 0.10 (0.1, 0.1) |
| Fresh Epinephrine     | Fresh | 0 | 0.40 (0.4, 0.4) |
| Fresh Epinephrine     | Fresh | + | 0.25 (0.2, 0.3) |

other hand, epinephrine added to medium from control cells (incubated for 20 min without hormone) induced a 300% increase in the cyclic AMP level of fresh cells.

DISCUSSION

Because of the rapidity with which the rate of lipolysis increases from basal to maximal after exposure of fat cells to epinephrine (or ACTH) and the equally rapid decline in the rate of lipolysis to basal level when action of the hormone is terminated, it is not technically feasible to show that changes in cyclic AMP concentration precede changes in lipase activity. Further, there would appear to be no necessary relationship between the peak level of cyclic AMP attained between 4 and 7 min after addition of epinephrine or ACTH and the rate of lipolysis (presumably a direct reflection of the amount of active lipase), since the rate of lipolysis is established well before this level is reached and is maintained unchanged while the cyclic AMP level then falls.

Butcher et al. (1) presented log-dose response curves for epinephrine and ACTH by using cyclic AMP levels measured after 10 min of incubation of fat cells with 1 mm caffeine plus hormone. Most of these levels were, however, greatly in excess of the peak concentrations produced by maximally effective concentrations of hormones in the absence of phosphodiesterase inhibition, and were in a range over which rates of lipolysis are maximal. The authors found it impossible to correlate these cyclic AMP levels with rates of lipolysis. We were interested to note, however, that the dose response curves in Reference 1 are almost superimposable on curves that we had prepared by plotting initial rate of glycerol production (as percentage of maximal) against log of hormone concentration (in the absence of phosphodiesterase inhibition). Presumably the amount of cyclic AMP accumulated in 10 min when caffeine is present to inhibit phosphodiesterase is rather directly related to its rate of formation, i.e. to the level of adenyl cyclase activity. If so, it appears that the extent of the lipase activation is closely linked to the extent of cyclase activation, and that maintenance of maximal lipase activity requires maximal cyclase activity (in the absence of phosphodiesterase inhibition). In other words, although the range of concentrations over which intracellular cyclic AMP is limiting in stimulating lipolysis includes only a small part of the observable spectrum (1), in fact, lipase activity is limited by the level of adenyl cyclase activity over essentially its entire range (in the absence of methyl xanthines). Such close coupling is clearly related to the very short half-life (high turnover rate) of cyclic AMP in the fat cell which is evidenced by the magnitude of the difference between the rate of cyclic AMP accumulation in the presence of hormone alone and that observed when phosphodiesterase is partially inhibited by methyl xanthines, as well as by the rapidity with which the cyclic AMP level falls when hormone action is terminated.

The changes in cyclic AMP levels with time after hormone addition raise some important questions completely aside from their relationship (or lack thereof) to lipase activity. It is clear that cyclase activation occurs very quickly. This is observed also with broken cell preparations, in which there is no discernible delay in establishment of the hormone-stimulated rate of cyclic AMP formation (12, 13). Probably within 1 min after addition of hormones the concentration of cyclic AMP in the fat cell can rise sufficiently to produce a maximal level of lipase activity. If the rate of cyclic AMP formation then continued unchanged in the presence of the hormone, the concentration of nucleotide would be expected to rise until the rate of breakdown was equal to that of formation, and remain constant thereafter.

It seemed possible that in previous studies (1–3, 14) the secondary decline in cyclic AMP levels was specifically related to the presence of the phosphodiesterase inhibitor, that the level or effect of methyl xanthine was decreasing with time, or that the remarkably high levels of cyclic AMP attained under these conditions somehow induced this response. As reported above, however, the decline in cyclic AMP level after an initial rise occurred also in the absence of methyl xanthines when the maximal rise in cyclic AMP level may be 100 to 200% over the basal. A similar decrease in levels despite continued presence of hormone has been reported to occur in muscle (15) and in slices of cerebellum (16). In our studies this decline was clearly not due to decrease in the effective concentration of hormone. Cyclic AMP formation continued to be stimulated during this period, but whether the rate of formation of cyclic AMP remained maximal, i.e. equal to that during the first minutes after addition of the hormone, cannot be established. Accumulation of a prostaglandin, for example, could interfere with hormonal stimulation of cyclase activity. Alternatively, if cyclase remained fully activated, the rate of cyclic AMP formation might decline, due either to substrate limitation or to enzyme inhibition by some material accumulated during incubation with hormone.

It is equally possible that one of the sequelae of hormonal stimulation of adenyl cyclase activity leads to an increase in the rate of degradation of cyclic AMP. The experimental approach to this question is complicated by the fact that there appears to
be more than one cyclic nucleotide phosphodiesterase (more than one $K_m$) for cyclic AMP in fat cells (7). In addition, phosphodiesterase activity in the intact cell might be influenced (regulated) by the concentration of some intermediate, e.g., ATP, as suggested by Cheung (17), and no change in the amount or characteristics of enzyme assayed in homogenates would be evident. Thus far our attempts to show directly alterations in phosphodiesterase activity during incubation of cells with epinephrine have been unsuccessful.

Whatever the mechanism of the secondary decline in cyclic AMP level that occurs despite the presence of hormone, it probably also plays a part in producing attenuation of the rise in cyclic AMP levels observed when ACTH is added to cells previously exposed to epinephrine and then to propranolol. There appears to be something accumulated in the medium during incubation of cells with epinephrine that interferes with the effectiveness of the hormone in causing the initial accumulation of cyclic AMP in fresh cells. Whether this material has its effect on the rate of formation or of degradation of cyclic AMP cannot be decided at present. The physiological significance of what might be descriptively termed “refractoriness” or diminished capacity of the cell to accumulate cyclic AMP in response to a second addition of hormone is unknown. Kakiuchi and Rall (16) similarly observed that a second addition of norepinephrine (or histamine) to brain slices failed to produce a second increment in cyclic AMP levels. If this phenomenon is in fact produced by a substance accumulated in the medium during incubation in vitro, it may be of little importance for the cell in vivo. On the other hand, even if it is in a sense an artifact of the experimental situation, elucidation of its genesis may contribute to a better understanding of the mechanisms underlying regulation of intracellular cyclic AMP levels.

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