The Relationship between the Concentration of Adenosine 3':5'-Cyclic Monophosphate and the Anti-Lipolytic Action of Insulin in Isolated Rat Fat-Cells

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The relationship between cyclic AMP content and lipolysis, as measured by glycerol formation, was studied in isolated rat fat-cells. Inhibition of lipolysis by insulin in the presence of a low concentration of adrenaline was accompanied by little or no lowering of cyclic AMP content, measured after 15 min incubation. The time-course of cyclic AMP content after addition of adrenaline showed that the effect of insulin in lowering cyclic AMP content measured after 2-5 min was gradually lost over the next hour, mainly because of the fall in cyclic AMP content after an early peak in the presence of adrenaline alone. There was a 44% loss of immunoreactive insulin, from an initial concentration of 0.3 nM, during a 1 h incubation with fat-cells. Insulin did not affect partitioning of cyclic AMP between cells and incubation medium. When the correlation between cyclic AMP content and rate of lipolysis was investigated for a wide range of adrenaline concentrations, it was found that the lowering of cyclic AMP content by insulin was much less than that required to account for the amount of inhibition of lipolysis. It is concluded that inhibition of adrenaline-stimulated lipolysis by insulin involves factors in addition to a decrease in intracellular cyclic AMP concentration.

A great deal of evidence has indicated that the stimulation of lipolysis by various hormones in adipose tissue is mediated by activation of adenylate cyclase in the fat-cell membrane (Robison *et al.*, 1971). The consequent increase in the intracellular concentration of cyclic AMP is thought to activate a protein kinase which phosphorylates and activates triglyceride lipase (Huttunen & Steinberg, 1971; Soderling *et al.*, 1973), though the precise sequence of events and the possible role of factors other than cyclic AMP, such as Ca^{2+} ions (Efendic *et al.*, 1970; Werner *et al.*, 1972; Exton *et al.*, 1972; Siddle & Hales, 1972), is not yet fully understood.

Insulin inhibits hormone-stimulated lipolysis in adipose tissue. This action is exerted on triglyceride hydrolysis, and is independent of effects of insulin on glucose uptake and fatty acid esterification (Jungas & Ball, 1963). The mechanism whereby insulin inhibits activation of lipolysis by hormones such as adrenaline has not been established. Under suitable conditions, insulin decreases the rise in cyclic AMP concentration that is produced by catecholamines in adipose tissue (Butcher *et al.*, 1968; Kuo & De Renzo, 1969; Manganiello *et al.*, 1971). Effects of insulin in inhibiting adenylate cyclase (Illiano & Cuatrecasas, 1972; Hepp & Renner, 1972) and stimulating cyclic nucleotide phosphodiesterase (Loten & Sneyd, 1970; Vaughan, 1972) from adipose tissue have been

reported. However, these results have not always been reproduced by other workers, and the mechanism whereby insulin lowers amounts of cyclic AMP in tissues remains a subject of controversy (Schönhöfer *et al.*, 1972). Even though insulin can lower adipose-tissue cyclic AMP content in the presence of lipolytic hormones, it has not been firmly established that this effect could explain all or even any of its actions in adipose tissue. It has been suggested that there is a relationship between the effects of insulin on cyclic AMP content and on such processes as glycogenolysis and lipolysis (Jungas, 1966; Park *et al.*, 1967; Butcher *et al.*, 1968) or protein kinase activation (Soderling *et al.*, 1973). However, Fain & Rosenberg (1972) and Khoo *et al.* (1973) showed a substantial inhibition of lipolysis by insulin which was accompanied by little or no lowering of cyclic AMP concentration. During the preparation of the present manuscript, Kono & Barham (1973) reported that there was a difference in the relationship between cyclic AMP content and lipolytic rate in the presence and absence of insulin when adrenocorticotrophic hormone was used as the lipolytic stimulus, but not if lipolysis was stimulated by caffeine. It is possible therefore that insulin exerts effects on the process of lipase activation other than by lowering cyclic AMP concentration.

The present studies were undertaken to examine

carefully the correlation between cyclic AMP concentration and adrenaline-stimulated lipolysis in the presence and absence of insulin, with fat-cells isolated from rat. In this way it was hoped to test whether the amount by which cyclic AMP concentration is lowered by insulin can account for the anti-lipolytic effect of the hormone.

Experimental

Materials

Chemicals. [8-³H]Adenosine 3':5'-cyclic monophosphate (20–25 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; crystalline ox insulin (24 units/mg) was from Burroughs Wellcome and Co., Dartford, Kent, U.K.; Repelcote, for siliconization of glassware, and adrenaline were from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; collagenase was from Worthington Biochemicals Corp., Freehold, N.J., U.S.A.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.

Animals. Male Wistar rats (120–140 g), bred in the laboratory or obtained from Ralph Tuck Ltd., Rayleigh, Essex, U.K., and given free access to food and water, were used in all experiments.

Methods

Preparation and incubation of fat-cells. Isolated fat-cells were prepared from epididymal adipose tissue by the method of Rodbell (1964). The standard incubation medium was Krebs–Ringer bicarbonate medium containing 1.3 mM-Ca²⁺ (Cohen, 1957) and 4% (w/v) bovine serum albumin but no glucose. The albumin was made up as a 30% (w/v) solution in Krebs–Ringer medium and dialysed overnight at 4°C against a tenfold excess of the medium before dilution to the final concentration. Media were gassed with O₂+CO₂ (95:5) and maintained at 37°C.

Epididymal fat-pads removed from about six rats were incubated with shaking (120/min) in 10 ml of medium containing 15 mg of collagenase (approx. 140 units/mg) for 30–45 min. Cells were washed with 3×10 ml of the standard medium and finally resuspended in this medium. The isolated fat-cells were prepared in plastic or silicone-treated glass vessels and pipetted with silicone-treated glass wide-bore pipettes.

Incubations of cells were carried out with 1 ml of suspension in capped polythene tubes (75 mm×25 mm). The dry weight of cells, measured by the difference in weight between 1 ml of cell suspension and 1 ml of medium dried overnight at 70°C, was used as the basis for expression of results.

Additions to the cells. Adrenaline solutions were made up freshly for each experiment by dissolving adrenaline in a minimum amount of 0.1 M-HCl and

diluting to 100 times the required final concentration in water.

Insulin was dissolved in 0.03 M-HCl at a concentration of 1 mg/ml and stored frozen in batches. A solution at 100 times the required final concentration was freshly prepared from frozen stock by diluting in incubation medium.

Additions were made to incubation vessels in 10 µl amounts before the cell suspension was pipetted out. When both hormones were used, they were therefore present simultaneously from the start of the cell incubation.

Extraction of cells. Incubations were terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and then the acid was removed by extracting with 4×5 ml of diethyl ether. Samples of this extract were used directly for assay of glycerol and were freeze-dried and redissolved in one-third their original volume of 100 mM-potassium phosphate buffer, pH 7.0, for assay of total cyclic AMP. This procedure was modified as follows when cyclic AMP was to be assayed separately in cells and incubation medium. Cell suspension (0.7 ml) was removed to a plastic centrifuge tube and centrifuged at approx. 200 g for 15 s. Infranant (0.5 ml) was removed and 10% trichloroacetic acid (0.5 ml) was added to both cells and infranant. The volume of the 'cell' extract was made up to 1 ml with incubation medium, and the volumes of both 'cell' and 'medium' extracts were then made up to 2 ml with water. After centrifugation and ether extraction, portions of these extracts were freeze-dried. These were redissolved in one-sixth their original volume of 100 mM-potassium phosphate buffer, pH 7.0, and samples taken for assay of cyclic AMP. Cyclic AMP content was calculated separately for cells and incubation medium after allowance had been made for medium present in the 'cell' extract, this being 0.2 ml (the remaining suspension volume) minus the cell volume, calculated assuming a specific gravity of 0.85 for triglyceride. A possible 10% uncertainty in cell volume was a negligible contribution to the volume of medium present in the 'cell' extract, and the cyclic AMP in the medium of the 'cell' extract was itself less than 10% of the total cyclic AMP in this extract.

Glycerol assay. Glycerol was assayed by the method of Garland & Randle (1962). Glycerol release (µmol/g dry wt.) was taken as a measure of lipolysis. The maximum rate of re-utilization of glycerol by fat-cells is very low compared with rates of release measured in the present work (Robinson & Newsholme, 1967).

Cyclic AMP assay. Cyclic AMP was measured by radioimmunoassay as previously described (Steiner *et al.*, 1969, as modified by Siddle *et al.*, 1973). Standard solutions taken through the procedure for fat-cell extractions were used, and gave a standard

Fig. 1. Dose response of insulin on lipolysis and cyclic AMP content

Fat-cells were incubated with adrenaline ($0.55 \mu\text{M}$) plus insulin. Each incubation vessel contained 1 ml of cell suspension at a concentration of 43 mg dry wt./ml. After 15 min the incubation was terminated by addition of trichloroacetic acid. The concentration of glycerol (a) and cyclic AMP (b) in the cell extract was measured as described in the text. Basal cell cyclic AMP content (no adrenaline or insulin added) was 0.12 ± 0.01 nmol/g dry wt. Maximum glycerol release ($0.55 \mu\text{M}$ -adrenaline plus 1 mM-theophylline) was 16.4 ± 0.5 $\mu\text{mol}/15$ min per g dry wt. Vertical bars represent means $\pm$ S.E.M. of four observations.

curve superimposable on that set up in phosphate buffer.

Insulin assay. Insulin was assayed in fat-cell incubation media by method C of Hales & Randle (1963).

Results

The effect of insulin on lipolysis and cyclic AMP content in the presence of a submaximal concentration of adrenaline ($0.55 \mu\text{M}$) is shown in Fig. 1. The inhibition of adrenaline-stimulated lipolysis by increasing concentrations of insulin was not in this case accompanied by any significant lowering of the elevated content of cyclic AMP, measurements of both glycerol formation and cyclic AMP content being made after 15 min incubation. An insulin concentration of 0.35 nM ($50 \mu\text{units}/\text{ml}$) produced a maximal inhibition of lipolysis and this concentration was used in further experiments. There was some indication that high insulin concentrations (35 nM)

were less effective at inhibiting lipolysis, as previously reported (Hales *et al.*, 1968; Solomon *et al.*, 1970; Kono, 1972).

The effect of insulin was investigated over a 1 h incubation period to test whether measurements made at a single time-point give a valid measure of initial cyclic AMP concentration and rate of lipolysis (Fig. 2). Adrenaline was used at a concentration ($5.5 \mu\text{M}$) that produced maximal activation of lipolysis, and insulin was used at 0.3 nM concentration. A steady rate of lipolysis was established within 5 min and then maintained up to 60 min, both in the presence and absence of insulin. The inhibition of lipolysis by insulin was accompanied by a lowering of the peak of cyclic AMP content reached after 5 min of incubation. In the presence of adrenaline alone, the cyclic AMP content declined after the peak, though less rapidly than observed by some

Fig. 2. Time-course of glycerol release, cyclic AMP content and insulin concentration

Cells were incubated for various periods up to 1 h. Cell concentration was 28 mg dry wt./ml. Incubations were terminated by addition of trichloroacetic acid for measurement of glycerol formation (a), or cyclic AMP content (b), or by centrifugation and removal of infranatant for measurement of insulin concentration (c). ■, Basal (no additions); ○, +adrenaline ($5.5 \mu\text{M}$); ●, +adrenaline ($5.5 \mu\text{M}$) and insulin (0.3 nM initial concentration). Vertical bars represent means $\pm$ S.E.M. of three observations.

Fig. 3. Partitioning of cyclic AMP between cells and incubation medium

After incubation cells and medium were separated by centrifugation and cyclic AMP was measured in the two fractions, for calculation of total cyclic AMP in cells plus medium (a) and cyclic AMP in cells as a fraction of the total (b). Cell concentration was 27 mg dry wt./ml. ○, +Adrenaline (5.5 μM). Glycerol formation was 45.7 ± 0.7 μmol/h per g dry wt. ●, +Adrenaline (5.5 μM) and insulin (0.35 nM). Glycerol formation was 14.7 ± 0.9 μmol/h per g dry wt. Vertical bars represent means $\pm$ S.E.M. of three observations.

workers (Ho & Sutherland, 1971; Manganiello *et al.*, 1971). However, in the presence of adrenaline plus insulin, the cyclic AMP content remained fairly steady over an hour after the initial rise, or in some experiments even showed a further gradual increase. Both in the presence and absence of insulin, the relationship between cyclic AMP content and lipolysis will therefore depend on the time at which measurements are made.

The insulin concentration remaining during the experiment of Fig. 2 was measured in samples of infranatant from incubations terminated by centrifugation. There was a loss of 44% of the immunoreactive insulin during the 1 h incubation (Fig. 2).

It is possible that in some experiments a greater rate of insulin destruction occurred, which lowered the insulin concentration sufficiently below the maximally effective amount for a lessening of the effects of the hormone to be observed as the incubation time increased. The nature of the insulin-degrading system is uncertain, and it may have involved specific cellular mechanisms (Crofford *et al.*, 1972), residual collagenase adhering to cells in spite of washing, or enzymes released from broken cells.

It is known that some of the cyclic AMP produced when fat-cells are exposed to catecholamines appears extracellularly (Kuo & De Renzo, 1969). The relative partitioning of cyclic AMP between cells and incubation medium was investigated in the presence and absence of insulin (Fig. 3). The total cyclic AMP content was calculated in this case from the contents measured separately in cells and medium. The effect of the procedure to separate cells and medium on the relative and absolute cyclic AMP contents is uncertain. However, cyclic AMP in the cells accounted for 60–70% of the total (and up to 80% in some experiments), at times up to 30 min both in the presence and absence of insulin. The higher value for total cyclic AMP in the presence of insulin at 1 h was accompanied by an increased proportion of cyclic AMP in the medium. It was concluded that at least for time-points up to 30 min, measurement of total cell suspension cyclic AMP provides a convenient and valid index of cell cyclic AMP content, and that insulin has no significant effect on partitioning of cyclic AMP between cells and medium.

The relationship between lipolysis and total cyclic AMP content was tested with several concentrations of adrenaline with and without insulin, measurements being made after 15 min incubation (Table 1). A

Table 1. Effect of adrenaline and insulin on glycerol formation and cyclic AMP content

Fat-cells were incubated with hormones at the concentrations shown. The cell concentration was 20 mg dry wt./ml with 1 ml of cell suspension in each incubation vessel. After 15 min incubations were terminated by addition of trichloroacetic acid. Glycerol and cyclic AMP were measured in the deproteinized extract as described in the text. Each value is the mean $\pm$ S.E.M. of four observations.

[Adrenaline] (μM)	[Insulin] (nM)	Amount of glycerol (μmol/g dry wt.)	Cyclic AMP content (nmol/g dry wt.)
0	0	0.3 ± 0.3	0.16 ± 0.01
0.28	0	8.0 ± 0.3	0.35 ± 0.02
0.28	0.35	0.8 ± 0.3	0.25 ± 0.01
0.55	0	11.1 ± 0.3	0.48 ± 0.02
0.55	0.35	1.1 ± 0.1	0.34 ± 0.01
5.5	0	13.3 ± 0.8	0.99 ± 0.03
5.5	0.35	5.1 ± 0.5	0.58 ± 0.03

Fig. 4. Relationship between cyclic AMP content and lipolysis in the presence and absence of insulin

Measurements were made on duplicate samples incubated with hormones for 5 min or for 15 min. Total cyclic AMP content of the cell suspension was measured at both times, and the rate of glycerol formation over the period 5–15 min was calculated from the means of the values for glycerol concentration at these two times. Cell concentration was 23 mg dry wt./ml. Adrenaline was used at concentrations of 0.055–5.5 μM in the absence of insulin, or 0.55–55 μM when insulin (0.35 nM) was also present. With adrenaline alone: ●, cyclic AMP at 5 min; ○, cyclic AMP at 15 min. With adrenaline+insulin (0.35 nM): ■, cyclic AMP at 5 min; □, cyclic AMP at 15 min.

reversal of the expected relationship between lipolysis and cyclic AMP content is seen when the data for 0.28 and 0.55 μM -adrenaline are compared with those for 5.5 μM -adrenaline plus 0.35 nM-insulin. The latter conditions gave a lower glycerol release than the former but with a higher cyclic AMP content. The same pattern of results could be obtained if glucose (2 mM) was included in the incubation medium. The inhibition of adrenaline-stimulated lipolysis by insulin could not therefore be correlated with the lowering of elevated cyclic AMP content when measurements were made at a single time-point.

The relationship between lipolysis and cyclic AMP content was re-examined by using measurements made at both 5 and 15 min. The initial steady rate of lipolysis was calculated from the glycerol produced between these times. Adrenaline concentrations between 0.055 and 5.5 μM , or tenfold higher concentrations plus insulin (0.35 nM), were used to give a range of lipolytic rates. The rate of lipolysis was then plotted as a function of the total content of cyclic AMP (Fig. 4). In the presence of adrenaline alone, maximum lipolysis was obtained with a 2.5–3-fold increase in cyclic AMP concentration. However, a

different relationship was obtained when insulin was present and cyclic AMP could then be increased fourfold without lipolysis reaching half-maximum rates. The difference in relationship between cyclic AMP content and lipolysis in the presence and absence of insulin was marked if the cyclic AMP content at 5 min was considered but was even more apparent on the basis of cyclic AMP content at 15 min.

Discussion

A problem in interpreting the correlation between cyclic AMP content and lipolytic rates is the possibility that a considerable portion of cyclic AMP in the cell is bound or metabolically inactive (Kuo & De Renzo, 1969; Robison *et al.*, 1971). This is suggested by the fact that the basal cell content of cyclic AMP in several tissues, if evenly distributed in cell water, would be much more than sufficient for full activation of the cyclic AMP-dependent protein kinases according to the properties of these enzymes as studied in cell-free systems (Park *et al.*, 1967; Robison *et al.*, 1971; Corbin *et al.*, 1973). The narrow range of cyclic AMP concentration effective in regulating the rate of lipolysis has also been interpreted as suggesting a high proportion of sequestered cyclic AMP (Robison *et al.*, 1971). The manner in which cyclic AMP might be sequestered is not known. It is unlikely that binding would be to regulatory (R) subunits of the protein kinase if regulatory and catalytic (C) subunits are present in equivalent amounts, as it is then difficult to see how a small fractional increase in cyclic AMP could produce the large metabolic effects that are believed to be mediated by increased kinase activity.

If the basal cell content of cyclic AMP is in some way compartmented, then it is possible that the increase in content in the presence of hormones also may not reflect a uniform increase in intracellular cyclic AMP concentration. Hence it could be argued that a difference in the relationship between cyclic AMP content and lipolysis in the presence and absence of insulin may reflect differences in the distribution of cyclic AMP. However, it is not clear how insulin might have selective effects on a pool of cyclic AMP to which protein kinase and triglyceride lipase respond, although the existence of several types of adenylate cyclase or phosphodiesterase would not rule out such a possibility. It is also conceivable that insulin might affect the distribution of cyclic AMP within the cell independently of effects on the total content of the nucleotide, but the results of Fig. 3 and those of Kuo & De Renzo (1969) show that there is no significant effect on partitioning of cyclic AMP between cells and incubation medium. It has recently been claimed by Soderling *et al.* (1973) that the effect of insulin on cyclic AMP-dependent protein kinase activity in fat-pads correlates with the effect

on cyclic AMP content, suggesting that insulin does not affect cyclic AMP distribution within the cell.

Fain & Rosenberg (1972) and Khoo *et al.* (1973) have observed an anti-lipolytic effect of insulin with little or no accompanying decrease in cyclic AMP content under suitable conditions. Jarett *et al.* (1972) similarly found that insulin could overcome the inhibition of protein synthesis caused by adreno-corticotrophic hormone or adrenaline without lowering the elevated cyclic AMP content, although the role of cyclic AMP in control of protein synthesis is still uncertain. Kono & Barham (1973) found a complex relationship between cyclic AMP content and the rate of lipolysis in the presence of insulin, depending on the nature and concentration of lipolytic stimulus and on the concentration of insulin. It was suggested that the effect of insulin (0.2 nM) in lowering the cellular content of cyclic AMP was large enough to account for its anti-lipolytic effect when caffeine was used as stimulus, or with other stimuli when cyclic AMP concentration was low, but not when determinations were made in the presence of nor-adrenaline (1 μ M) or adrenocorticotrophic hormone (10–100 nM), producing high concentrations of cyclic AMP. It has been shown in the present work that the amount by which a physiological insulin concentration (0.35 nM) lowers cyclic AMP content in isolated fat-cells is not sufficient to account for the inhibition of lipolysis when adrenaline at a wide range of concentrations is used as lipolytic stimulus (Table 1, Figs. 1 and 4). There was no indication that the response to insulin differed at low and high cyclic AMP concentrations (Fig. 4) as reported by Kono & Barham (1973).

If total cyclic AMP content gives a direct measure of the concentration of nucleotide effective in activating protein kinase and mediating effects on lipolysis, then cyclic AMP cannot be the only factor involved in the regulation of this process, since a given rate of lipolysis proceeds with a greater cell content of cyclic AMP in the presence of insulin than in its absence. The nature of other factors involved in the regulation of lipolysis is uncertain, though a role has been proposed for Ca^{2+} ions (Efendic *et al.*, 1970; Werner *et al.*, 1972; Siddle & Hales, 1972) and for cyclic GMP (Illiano *et al.*, 1973). Several effects of insulin in adipose tissue, such as those on protein synthesis (Jarett *et al.*, 1972), glucose transport (Robison *et al.*, 1971) and pyruvate dehydrogenase activity (Martin *et al.*, 1972), are apparently not directly mediated by the effect of insulin on cyclic AMP concentration. The simplest interpretation of the experiments reported in this paper is that the lowering of cyclic AMP concentration by insulin is also not the major means by which the hormone decreases the activation of triglyceride lipase in the presence of adrenaline. If there exists a single underlying mechanism for the diverse effects of insulin on

adipose-tissue metabolism, it seems unlikely that this directly involves cyclic AMP, and the effect of insulin on cyclic AMP content may be a subsidiary one in explaining any of the actions of the hormone.

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