The Effects of Insulin on Lipolysis Evoked by Cyclic AMP and Its Dibutyryl Analog* (33497)

H. MAURICE GOODMAN[1]

Department of Physiology, Harvard Medical School, Boston, Massachusetts

It now appears reasonably well established that hormone-induced lipolysis in adipose tissue is mediated by the cyclic nucleotide, adenosine 3′ 5′-monophosphate (CAMP) (1). However, attempts to induce lipolysis by adding CAMP directly to intact adipose cells or tissue segments have largely been unsuccessful except when the ionic composition of the incubation medium was manipulated (2) or when theophylline was present (3, 4). This failure was probably due to a combination of poor penetrability of CAMP and its rapid destruction by the enzyme CAMP phosphodiesterase. Consequently, the N^6-2′-*O*-dibutyryl analog of CAMP (DB CAMP) came into widespread experimental use since it allegedly penetrates cell membranes more readily and is less susceptible to enzymatic destruction than the natural cyclic nucleotide (5). In other respects, the biological activity of DBCAMP is thought to parallel that of the natural nucleotide. Recently, however, we have found that CAMP and DB CAMP differ profoundly in the susceptibility of their lipolytic effects to blockade by insulin. The present report describes these findings.

Materials and Methods: Adult male rats of the Holtzman strain were used for all experiments. They were fed Purina lab chow and water up to the time of sacrifice. Rats were killed by cervical dislocation. The epididymal fat was quickly excised and divided into 6 or 8 segments weighing 50–100 mg each. Only the thin, distal portions of the fat bodies were used. The tissues were randomly distributed among incubation vials which contained the various test substances. The incubation medium was Krebs–Ringer bicarbonate buffer and contained 40 mg/ml of bovine serum albumin (Armour, Fraction V) and 1 mg/ml of glucose. Theophylline (Nutritional Biochemicals), CAMP (Sigma Chemical Co.), DBCAMP (Calbiochem), insulin (Iletin, regular, Lilly) and imidazole (Calbiochem) were added at the concentrations indicated in the text. Incubations were carried out for 1 hr at 37° in sealed vials under an atmosphere of 95% O_2:5% CO_2. Release of glycerol into the medium served as a measure of lipolysis and was quantitated by the enzymatic procedure of Weiland (6).

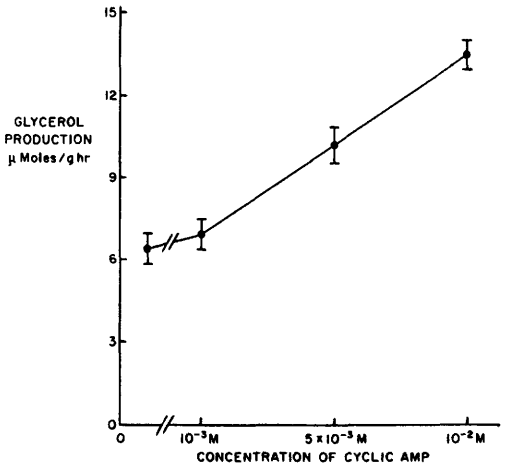

FIG. 1. The effects of CAMP on lipolysis in the presence of 0.3 mg/ml of theophylline. Each point represents the mean of 8 observations; the standard errors are indicated by the brackets.

Results. The addition of CAMP to segments of adipose tissue in the presence of 0.3 mg/ml (1.67×10^{-3} *M*) theophylline resulted in a concentration-dependent increase in glycerol production (Fig. 1). At a concentration of 10^{-3} *M*, CAMP produced no greater release of glycerol than did theophylline alone, but sizable increases in glycerol production were seen when the concentration of CAMP was raised to 5×10^{-3} or 10^{-2} *M*.

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TABLE I. The Effects of Insulin on Lipolysis in Response to Theophylline and CAMP.

Additions to medium	Glycerol production (μmoles/g/hr)	Increment due to CAMP
Control	2.04 ± 0.26[a]	
Theophylline (0.3 mg/ml)	5.15 ± 0.28	
Theophylline + CAMP (10^{-2} *M*)	9.63 ± 1.27	4.48 ± 1.38
Insulin (1 mU/ml)	2.15 ± 0.42	
Theophylline + insulin	4.01 ± 0.28	
Theophylline + insulin + CAMP	4.59 ± 0.40	0.58 ± 0.57[b]

[a] Mean ± SEM; 8 observations.
[b] Significant reduction due to insulin ($p < 0.02$).

Similar results were obtained in a separate experiment (Table I). Theophylline alone increased glycerol production, and this effect was further enhanced by the addition of 10^{-2} *M* CAMP. Insulin alone did not affect basal glycerol production, and only slightly decreased the response to theophylline but it completely abolished the lipolytic response to CAMP.

TABLE II. The Effects of Insulin and Theophylline on Lipolysis in Response to DBCAMP.

Additions to medium	Glycerol production (μmoles/g/hr)	Increment due to DBCAMP
Control	1.12 ± 0.17[a]	
DBCAMP (10^{-3} *M*)	3.17 ± 0.44[d]	2.05 ± 0.40[d]
Insulin (1 mU/ml)	0.90 ± 0.16	
DBCAMP + insulin	3.36 ± 0.41[d]	2.47 ± 0.35[d]
Theophylline (0.3 mg/ml)	5.32 ± 0.39	
Theophylline + DBCAMP	10.37 ± 1.88[d]	5.27 ± 1.52[b,d]
Theophylline + insulin	3.45 ± 0.48	
Theophylline + insulin + DBCAMP	13.88 ± 1.06	9.72 ± 1.18[c,d]

[a] Mean ± SEM; 8 observations.
[b] Significantly greater increment than that due to DBCAMP alone ($p < .05$).
[c] Significantly greater increment than that due to theophylline + DBCAMP ($p < .05$).
[d] Significant lipolytic effect of DBCAMP ($p < .01$).

DBCAMP also caused a pronounced increase in glycerol production (Table II). Insulin failed to reduce lipolysis when added alone or in the presence of DBCAMP. Theophylline alone increased lipolysis and more than doubled the lipolytic response to DBCAMP. When added along with theophylline and the nucleotide, insulin greatly magnified the lipolytic response to DBCAMP. In some experiments (2 out of 4), insulin increased the output of glycerol caused by DBCAMP alone (Table III). This effect was seen even when glucose was omitted from the incubation medium.

TABLE III. The Effects of DBCAMP and Insulin on Lipolysis in the Presence and Absence of Glucose.[a]

Additions to medium	Glycerol production (μmoles/g/hr)	Increment due to DBCAMP
Glucose present		
Control	2.25 ± 0.44[b]	
DBCAMP (10^{-3} *M*)	3.76 ± 0.44[d]	1.51 ± 0.31[d]
DBCAMP + insulin (1 mU/ml)	5.09 ± 0.48[d]	2.83 ± 0.41[c,d]
Glucose absent		
Control	1.52 ± 0.43	
DBCAMP	3.13 ± 0.43[d]	1.61 ± 0.44[d]
DBCAMP + insulin	4.99 ± 0.55[d]	3.46 ± 0.55[c,d]

[a] (1 mg/ml).
[b] Mean ± SEM; 8 observations.
[c] Significant increase due to insulin ($p < .05$).
[d] Significant lipolytic effect of DBCAMP ($p < .05$).

Imidazole, which is thought to activate CAMP phosphodiesterase, the enzyme which inactivates CAMP (7), slightly reduced lipolysis caused by theophylline. Like insulin, imidazole completely abolished the lipolytic response to CAMP. Unlike insulin, however, imidazole also markedly reduced lipolysis in response to DBCAMP (Table IV).

Discussion: Consistent lipolytic effects of CAMP were obtained when the nucleotide was added in the presence of submaximal amounts of theophylline. Similar observations

TABLE IV. The Effects of Imidazole on Lipolysis in Response to Theophylline plus CAMP and to DBCAMP.

Additions to medium	Glycerol production (μmoles/g/hr)	Increment due to cyclic nucleotide
Control	1.47 ± 0.14[a]	
Theophylline (0.3 mg/ml)	4.67 ± 0.41	
Theophylline + CAMP (10^{-2} *M*)	8.97 ± 0.57	4.30 ± 0.56
Imidazole + theophylline	3.18 ± 0.47[b]	
Imidazole + theophylline + CAMP	2.89 ± 0.34	−0.29 ± 0.12[b]
DBCAMP (10^{-3} *M*)	5.63 ± 0.85	4.15 ± 0.52
Imidazole (10 mg/ml)	1.72 ± 0.48	
Imidazole + DBCAMP	2.80 ± 0.89	1.08 ± 0.05[b]

[a] Mean ± SEM; 7 observations.
[b] Significant reduction due to imidazole.

were made by Weiss *et al.* (4) and by Vaughan (3), although in the experiments of Mosinger and Vaughan (2), CAMP appeared to reduce lipolysis when added along with very low concentrations of theophylline. The concentration of CAMP required for lipolysis (10^{-3}–10^{-2}) is high compared with that actually found by direct analysis of CAMP in adipose tissue. Butcher and Sutherland (1) reported that segments of epididymal fat contained about 10 μmoles $\times$ 10^{-4}/g after incubation with epinephrine and caffeine. Assuming that all of the CAMP was distributed uniformly in intracellular water, and that intracellular water is about 5% of the wet weight of epididymal fat (8, 9), then the intracellular concentration of CAMP under these circumstances was 10 μmoles $\times$ 10^{-4} per 50 μl of intracellular water, or 2 $\times$ 10^{-5} *M*. More recent measurements by Butcher *et al.* (10) using isolated fat cells indicated that CAMP levels may rise as high as 10,000 pmoles/g of dry weight after incubation with epinephrine and caffeine. If the same ratio of intracellular water to dry weight holds for cells as for tissues, 1 g of dry weight should be the equivalent of 62 μl of intracellular water. The concentration of CAMP in the intracellular water of the fat cells would then be of the order of 1.6 $\times$ 10^{-4} *M*. In the absence of caffeine the concentration of CAMP was one fifth to one tenth as high, and in unstimulated cells the concentration of CAMP was about 10^{-5} *M*. The concentration of exogenously applied CAMP required for lipolysis is thus only about 500 times higher than the basal level. If the distribution of CAMP in intracellular water is nonuniform, this factor may be even lower. When consideration is given to possible difficulties in permeability and rapid enzymatic destruction of CAMP, the magnitude of the extracellular concentration of CAMP needed for lipolysis does not seem unreasonably high.

Insulin abolished lipolysis in response to CAMP both in the present studies and in the experiments of Mosinger and Vaughan (2), in which lipolytic effects of CAMP were obtained by manipulating the ionic composition of the incubation medium. Both the mechanism and the physiological significance of this effect of insulin are unknown. Mosinger and Vaughan suggested that effects of insulin on ion movement might be responsible for the decreased lipolytic response to CAMP. It is also possible that insulin decreased the rate of entry of CAMP into adipose cells. Another explanation for this effect of insulin might be that insulin hastens the destruction of CAMP by activating the enzyme CAMP phosphodiesterase. Insulin reputedly stimulates this enzyme in liver and muscle (11). Furthermore, Posternak *et al.* (5) claimed that dibutyryl CAMP was resistant to phosphodiesterase, a fact which might be invoked to explain, at least partially, the differences seen in the effects of insulin on CAMP and DBCAMP-induced lipolysis. However, the finding first reported by Aulich *et al.* (12) that theophylline potentiates the lipolytic action of DBCAMP provided indirect evidence that DBCAMP is degradable by phosphodiesterase. Furthermore, Blecher (13) showed that DBCAMP is a substrate for phosphodiesterase and that no stimulation of this enzyme was demonstrable when insulin was added directly to the assay system

or when phosphodiesterase was measured in homogenates of adipose cells which had been preincubated with insulin. The present findings with imidazole add further indirect support to these conclusions. Imidazole, which activated purified phosphodiesterase isolated from homogenates of beef heart (7) reduced lipolysis in response to both CAMP and DB CAMP. If insulin prevented lipolysis in response to CAMP by activating phosphodiesterase, it should also have decreased lipolysis in response to DBCAMP.

The paradoxical finding that insulin may enhance the lipolytic action of DBCAMP in some experiments was not reported previously, although others (13, 14) reported that insulin does not antagonize the lipolytic effects of DBCAMP. Current information is insufficient to explain how insulin sometimes enhances the lipolytic response to DBCAMP or why this effect is not always seen. Apparently it is not secondary to increased metabolism of glucose, for insulin increased the lipolytic effects of DBCAMP even when glucose was omitted from the incubation medium.

The present results indicate that CAMP and DBCAMP may behave differently under some circumstances. Although the mechanisms for the effects noted are unknown, and although their physiological significance remains to be established, these findings underscore the need for caution in drawing physiological conclusions based on findings with DBCAMP alone.

Summary. Glycerol production by segments of epididymal fat from normal rats was used as a measure of lipolysis. Theophylline (0.3 mg/ml) produced a striking increase in lipolysis. Further addition of cyclic adenosine 3′, 5′-monophosphate (CAMP) at a concentration of 10^{-2} *M* caused an even greater increase in glycerol production. Insulin (1 mU/ml) slightly reduced the lipolytic effects of theophylline and completely abolished lipolysis in response to CAMP. The dibutyryl analog of CAMP (10^{-3} *M*) also increased lipolysis and its effects were also potentiated by theophylline. Insulin failed to reduce and in some experiments even enhanced the lipolytic action of dibutyryl CAMP. Imidazole reduced lipolysis in response to both nucleotides. The findings indicate that CAMP and its dibutyryl analog may behave quite differently under some circumstances and underscore the need for caution in drawing physiological conclusions based on findings with DBCAMP alone.

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1. Butcher, R. W. and Sutherland, E. W., Ann. N. Y. Acad. Sci. **139**, 849 (1967).

2. Mosinger, B. and Vaughan, M., Biochim. Biophys. Acta **144**, 569 (1967).

3. Vaughan, M., Ann. N. Y. Acad. Sci. **139**, 849 (1967).

4. Weiss, B., Davies, J. I., and Brodie, B. B., Biochem. Pharm. **15**, 1553 (1966).

5. Posternak, T., Sutherland, E. W., and Henion, W. F., Biochim. Biophys. Acta **65**, 558 (1962).

6. Weiland, O., Biochem. Z. **329**, 313 (1957).

7. Butcher, R. W. and Sutherland, E. W., J. Biol. Chem. **237**, 1244 (1962).

8. Crofford, O. B. and Renold, A. E., J. Biol. Chem. **240**, 3237 (1965).

9. Goodman, H. M., Am. J. Physiol. **211**, 815 (1966).

10. Butcher, R. W., Baird, C. E., and Sutherland, E. W., J. Biol. Chem. **243**, 1705 (1968).

11. Schultz, G., Senft, G., and Muske, K., Naturwissenschaften **53**, 529 (1966).

12. Aulich, A., Stock, K., and Westermann, E., Life Sci. **6**, 929 (1967).

13. Blecher, M. Federation Proc. **27**, 495 (1968).

14. Peterson, M. J., Patterson, C., and Ashmore, J., Life Sci. **7**, 551 (1968).

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