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EVIDENCE FOR A ROLE OF ADENOSINE 3′,5′-MONOPHOSPHATE IN ADIPOSE TISSUE LIPOLYSIS

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Abstract—The nature of the processes whereby the mobilization of free fatty acids (FFA) is regulated by the sympathetic nervous system was examined from the lipolytic responses of isolated fat cells from rat epididymal adipose tissue. The production of FFA and glycerol is increased by norepinephrine, which stimulates the synthesis of adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP), and by theophylline, which inhibits the metabolism of the nucleotide. Since the maximal response to norepinephrine is only about one third that of theophylline, it may be inferred that the effects of the catecholamine are limited by the capacity of adenyl cyclase and not by that of the lipase system.

Direct evidence shows that cyclic 3′,5′-AMP can increase lipolysis in intact fat cells. Thus, the nucleotide itself produces little lipolysis but, together with small amounts of theophylline, causes effects similar to those elicited by maximally effective concentrations of theophylline.

The optimum responses to ACTH and norepinephrine are almost the same, though on a molar basis ACTH is 100 times as active as norepinephrine. Since theophylline potentiates the substances to about the same degree, it may be inferred that the lipolytic effects of both hormones are mediated through cyclic 3′,-5′-AMP.

SINCE the mammalian organism can respond swiftly to environmental changes, the activity of certain enzymes must be under the direct control of the nervous system through neurohormones released at nerve endings. A crucial problem in biology is the nature of the mechanisms by which these enzymes are stimulated by neurohormones.

An important clue to this problem was the discovery by Sutherland and Rall[1] that the breakdown of glycogen is regulated by sympathetic neurohormones which trigger the conversion of phosphorylase *b* to the active *a* form through the formation of adenosine 3′, 5′-monophosphate (cyclic 3′, 5′-AMP). These authors demonstrated that cyclic 3′, 5′-AMP is present in most mammalian tissues, and suggested that this nucleotide might mediate other sympathetic responses.

Mounting evidence that the sympathetic nervous system has an important role in the mobilization of free fatty acids (FFA)[2] has raised the possibility that cyclic 3′,5′-AMP is involved in the hydrolysis of triglycerides to FFA. Indications of this were reported by Rizack,[3] who showed that the activity of lipase in adipose tissue homogenates is almost doubled by this nucleotide. In recent studies, cyclic 3′,5′-AMP has been closely implicated in the sympathetic control of triglyceride breakdown.

For example, Butcher *et al.*[4, 5] reported that the incubation of epinephrine with rat adipose tissue enhances the formation of cyclic 3′, 5′-AMP and FFA. In addition, studies from this laboratory[6–9] have demonstrated that lipolysis is stimulated in the living animal and *in vitro* by theophylline, a drug that blocks phosphodiesterase, the enzyme responsible for the inactivation of cyclic 3′,5′-AMP.[10] Moreover, the increased lipolytic activity in adipose tissue is correlated with the rise in cyclic 3′,5′-AMP levels.[8]

The present report describes experiments with isolated fat cells from the rat epididymal fat pad. The results show that cyclic 3′,5′-AMP exerts a lipolytic effect on the intact cell. A comparison of the effects evoked by norepinephrine, theophylline, and cyclic 3′,5′-AMP suggests that the rate of lipolysis depends on the level of the nucleotide. A preliminary account of these studies has been presented.[6]

MATERIALS AND METHODS

Isolated fat cells were prepared according to Rodbell[11] from epididymal fat pads of male Sprague-Dawley rats weighing about 200 g. The production of glycerol or FFA was used as a measure of lipolysis. Fat cells, equivalent to 30 mg tissue, were suspended in plastic vessels in 2·5 ml Krebs-Ringer phosphate buffer, pH 7·4, containing 5 per cent albumin. Incubations were carried out for 1 hr at 37° in a metabolic shaker. Free fatty acids in the suspension were determined by the method of Dole and Meinertz;[12] glycerol according to Lambert and Neish,[13] and triglycerides by the method of Butler *et al.*[14]

Phosphodiesterase was assayed by the method of Butcher and Sutherland,[10] adapted for rat epididymal fat pads. In this procedure, phosphodiesterase activity is determined from the rate at which cyclic 3′,5′-AMP is converted to 5′-AMP, and the latter substance is measured from the inorganic phosphate formed by the action of alkaline phosphatase. Fat pads were homogenized in 0·33 M sucrose, and centrifuged (2000 *g*, 40 min) at 0°. The supernatant fluid was adjusted to 0·5 saturation with ammonium sulfate, maintaining the pH at 7·4 with KOH. The precipitate was separated by centrifugation (8000 *g*, 60 min) and taken up in 10^{-3}M Tris buffer (pH 7·5) containing 10^{-3}M $MgSO_4$. The enzyme preparation was then dialyzed for 24 hr in Visking casing against three changes of Tris buffer, centrifuged, and stored at −20° until used. Incubation mixture for enzyme assay consisted of cyclic 3′,5′-AMP (5×10^{-4}M), $MgSO_4$ ($3{\cdot}5 \times 10^{-3}$M), alkaline phosphatase (5×10^{-2} mg), and a suitable dilution of phosphodiesterase preparation (about 0·2 mg protein/ml) in Tris buffer (4×10^{-2}M), pH 8·0, to final volume of 2 ml. The reaction was carried out for 20 min at 37° in a metabolic shaker, stopped by the addition of 0·2 ml 55% trichloroacetic acid, and the inorganic phosphate measured in supernatant fluid.[15]

Materials. Chemicals used in this study and their sources are as follows: bovine albumin (fatty acid poor), from Gallard-Schlesinger; collagenase, Worthington Biochemical Corp.; *Escherichia coli* alkaline phosphatase type III, Sigma Chemical Co.; Acthar lyophilized corticotropin, Armour Pharmaceutical Co.; l-norepinephrine bitartrate, Winthrop Laboratories; and adenosine 3′,5′-cyclic phosphate, Nutritional Biochemicals Corp.

RESULTS

Lipolytic effects of norepinephrine, ACTH, *and theophylline*

In the absence of drug, the lipolytic activity of fat cells, as measured by glycerol or FFA production, was negligible. The effects of sympathetic stimulation were determined from the responses to norepinephrine (Fig. 1). These responses were dependent on the concentration of the catecholamine and were maximal at about 10^{-6}M. At higher concentrations of amine, the response decreased, as previously noted by Wenke *et al.*[13]

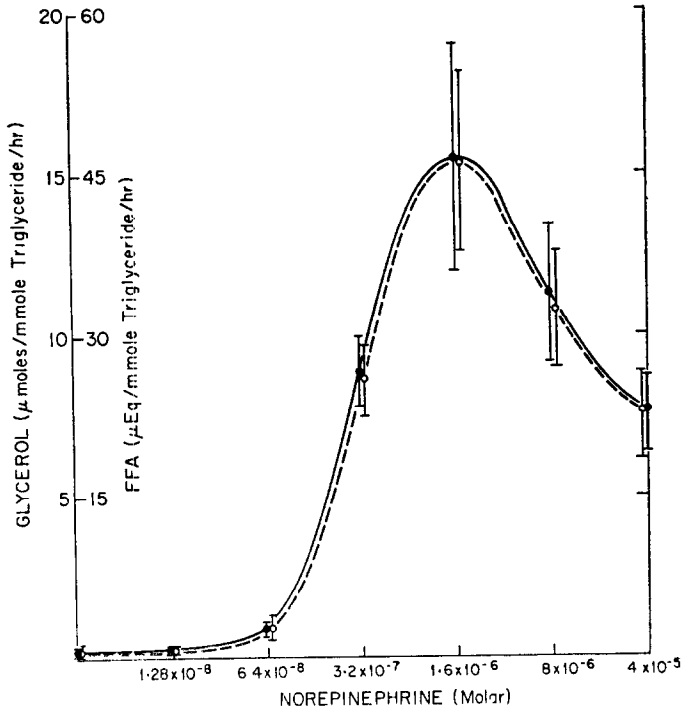

FIG. 1. Lipolytic effect of norepinephrine on isolated fat cells. Fat cells were incubated with norepinephrine for 1 hr. FFA, glycerol, and triglyceride were determined as described in Methods. For all figures: each point represents mean value of four experiments; vertical bars represent standard error of the mean; O - - - - - - - - - O FFA; ●———● glycerol.

From the responses of fat cells to various concentrations of ACTH (Fig. 2), it may be concluded that the maximal effect of the polypeptide was about the same as that of norepinephrine. In these experiments, however, the response to ACTH did not decline at high concentrations.

Figure 3 shows the lipolytic responses of fat cells to theophylline. The maximal effect was attained at about 10^{-2}M and was about three times that evoked by norepinephrine or ACTH. For each concentration of theophylline, as well as norepinephrine and ACTH, the FFA : glycerol ratio was essentially 3 : 1, indicating that reesterification of FFA was negligible.

The possibility was investigated that the lipolytic action of theophylline was associated with a decreased destruction of cyclic 3′,5′-AMP. Table 1 shows that the rate of

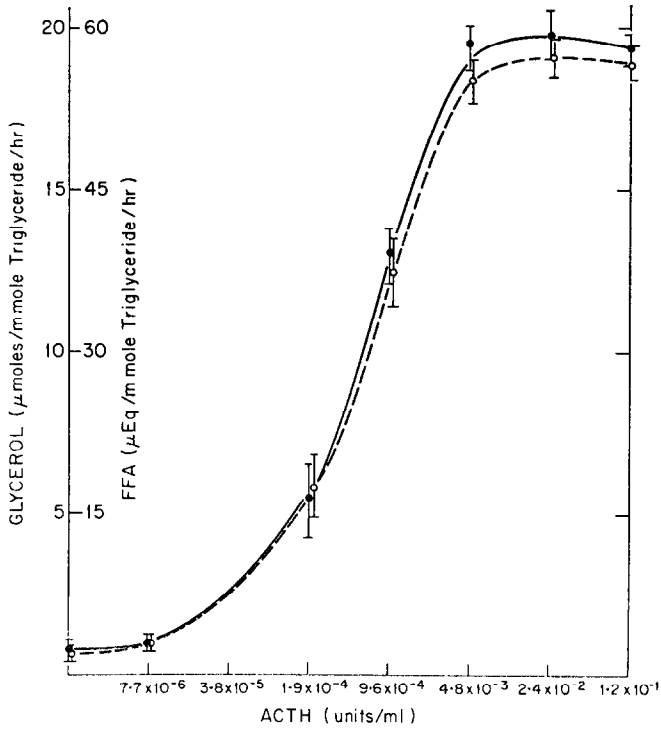

FIG. 2. Lipolytic effect of ACTH on isolated fat cells. Fat cells were incubated with ACTH for 1 hr. FFA, glycerol, and triglyceride were determined as described in Methods.

glycerol formation in the presence of theophylline was roughly correlated with the degree of phosphodiesterase inhibition.

Potentiation of responses to norepinephrine and ACTH *by theophylline*

If norepinephrine acts by increasing the synthesis of cyclic 3′,5′-AMP, and theophylline by protecting it from inactivation, the two substances should exert more than additive effects. At 4×10^{-4} M, theophylline produced only a slight release of glycerol, but this concentration of drug together with norepinephrine ($3{\cdot}2 \times 10^{-7}$ M) produced much more lipolysis than did maximally effective concentrations of norepinephrine (Table 2). However, the greatest response to the drug combination did not exceed that elicited by high concentrations of theophylline alone (1×10^{-2} M). Similarly, theophylline potentiated the lipolytic action of ACTH, but again the maximal response of the drug combination did not exceed that of theophylline alone (Table 3).

Lipolytic effects of cyclic 3′,5′-AMP

Studies were then carried out to see whether cyclic 3′,5′-AMP by itself could produce a lipolytic effect in intact cells. High concentrations of the nucleotide elicited only a slight lipolytic response, but this was greatly enhanced by theophylline in a concentration (4×10^{-4} M) that had only a slight effect by itself. Again the maximal

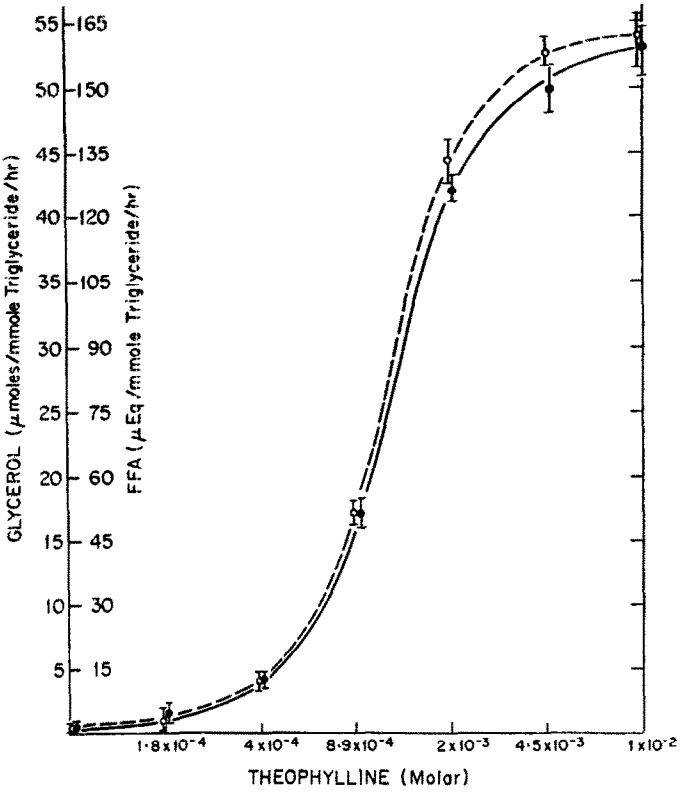

FIG. 3. Lipolytic effect of theophylline on isolated fat cells. Fat cells were incubated with theophylline for 1 hr. FFA, glycerol, and triglyceride were determined as described in Methods.

TABLE 1. EFFECTS OF THEOPHYLLINE ON BLOCKADE OF PHOSPHODIESTERASE AND ON GLYCEROL OUTPUT OF ISOLATED FAT CELLS

Concentration of theophylline (M)	Phosphodiesterase activity*	Inhibition of phosphodiesterase (%)	Glycerol† (μmole/m-mole triglyceride/hr)
	379 ± 8	0	0·5
1×10^{-4}	364 ± 14	4	1·0
$3{\cdot}2 \times 10^{-4}$	347 ± 12	8	4·3
1×10^{-3}	254 ± 12	33	21
$3{\cdot}2 \times 10^{-3}$	200 ± 3	47	48
1×10^{-2}	95 ± 7	75	53

Phosphodiesterase, prepared from rat adipose tissue, was incubated with theophylline. Cyclic 3′,5′-AMP was added and enzyme activity determined as described in Methods. Each value is mean of four experiments.

* Micromoles cyclic 3′,5′-AMP hydrolyzed/g protein/hr ± S.E.

† Data taken from Fig. 3

TABLE 2. EFFECTS OF THEOPHYLLINE AND NOREPINEPHRINE ON GLYCEROL OUTPUT OF ISOLATED FAT CELLS

Additions	Glycerol (μmole/m-mole triglyceride/hr) $\pm$ S.E.
None	0·2 $\pm$ 0·1
Norepinephrine ($1{\cdot}3 \times 10^{-8}$M)	0·5 $\pm$ 0·2
Norepinephrine ($3{\cdot}2 \times 10^{-7}$M)	22 $\pm$ 3
Norepinephrine ($8{\cdot}0 \times 10^{-6}$M)	15 $\pm$ 2
Theophylline (4×10^{-4}M)	4·6 $\pm$ 1
Theophylline (1×10^{-2}M)	57 $\pm$ 2
NE ($1{\cdot}3 \times 10^{-8}$M) + theophylline (4×10^{-4}M)	33 $\pm$ 3
NE ($3{\cdot}2 \times 10^{-7}$M) + theophylline (4×10^{-4}M)	64 $\pm$ 3
NE ($8{\cdot}0 \times 10^{-6}$M) + theophylline (4×10^{-4}M)	62 $\pm$ 3
NE ($1{\cdot}3 \times 10^{-8}$M) + theophylline (1×10^{-2}M)	54 $\pm$ 3
NE ($3{\cdot}2 \times 10^{-7}$M) + theophylline (1×10^{-2}M)	58 $\pm$ 2
NE ($8{\cdot}0 \times 10^{-6}$M) + theophylline (1×10^{-2}M)	54 $\pm$ 2

Fat cells prepared from rat epididymal fat pad were incubated for 1 hr. Each value is the mean of four experiments.

TABLE 3. EFFECTS OF THEOPHYLLINE AND ACTH ON GLYCEROL OUTPUT OF ISOLATED FAT CELLS

Additions	Glycerol (μmole/m-mole triglyceride/hr) $\pm$ S.E.
None	0·2 $\pm$ 0·1
ACTH ($7{\cdot}7 \times 10^{-6}$ U/ml)	0·2 $\pm$ 0·4
ACTH ($1{\cdot}9 \times 10^{-4}$ U/ml)	0·7 $\pm$ 0·2
ACTH ($4{\cdot}8 \times 10^{-3}$ U/ml)	12 $\pm$ 2
Theophylline (4×10^{-4}M)	4·6 $\pm$ 1
Theophylline (1×10^{-2}M)	57 $\pm$ 2
ACTH ($7{\cdot}7 \times 10^{-6}$ U/ml) + theophylline (4×10^{-4}M)	7·4 $\pm$ 1
ACTH ($1{\cdot}9 \times 10^{-4}$ U/ml) + theophylline (4×10^{-4}M)	59 $\pm$ 1
ACTH ($4{\cdot}8 \times 10^{-3}$ U/ml) + theophylline (4×10^{-4}M)	64 $\pm$ 3
ACTH ($7{\cdot}7 \times 10^{-6}$ U/ml) + theophylline (1×10^{-2}M)	58 $\pm$ 1
ACTH ($1{\cdot}9 \times 10^{-4}$ U/ml) + theophylline (1×10^{-2}M)	56 $\pm$ 2
ACTH ($4{\cdot}8 \times 10^{-3}$ U/ml) + theophylline (1×10^{-2}M)	57 $\pm$ 2

Fat cells were incubated for 1 hr. Each value is the mean of four experiments.

response caused by large amounts of both theophylline and cyclic 3′,5′-AMP did not exceed that produced by theophylline alone (Table 4).

Further evidence that cyclic 3′,5′-AMP can stimulate lipolysis in fat cells was obtained with adenosine. By itself, this substance exerted no lipolytic action, but was capable of reducing the response to theophylline. This action was restored by the addition of cyclic 3′,5′-AMP (Table 5) but not by 5′-AMP, adenine, or cytidine.

TABLE 4. EFFECTS OF THEOPHYLLINE AND CYCLIC 3′,5′-AMP ON GLYCEROL OUTPUT OF ISOLATED FAT CELLS

Additions	Glycerol (μmole/m-mole triglyceride/hr) ± S.E.
None	0·2 ± 0·1
Cyclic AMP (5×10^{-3}M)	1·4 ± 0·3
Cyclic AMP (1×10^{-2}M)	6·6 ± 0·7
Theophylline (4×10^{-4}M)	4·5 ± 1·4
Theophylline (1×10^{-2}M)	35 ± 0·4
Cyclic AMP (5×10^{-3}M) + theophylline (4×10^{-4}M)	17 ± 1
Cyclic AMP (1×10^{-2}M) + theophylline (4×10^{-4}M)	42 ± 1
Cyclic AMP (5×10^{-3}M) + theophylline (1×10^{-2}M)	36 ± 1
Cyclic AMP (1×10^{-2}M) + theophylline (1×10^{-2}M)	34 ± 1

Fat cells were incubated for 1 hr. Each value represents the mean of four experiments.

TABLE 5. EFFECTS OF CYCLIC 3′,5′-AMP ON GLYCEROL OUTPUT OF ISOLATED FAT CELLS IN THE PRESENCE OF THEOPHYLLINE AND ADENOSINE

Additions	Glycerol (μmole/m-mole triglyceride/hr) ± S.E.
None	0·2 ± 0·1
Adenosine (10^{-3}M)	0·1 ± 0·1
Theophylline (10^{-2}M)	36 ± 1
Theophylline (10^{-2}M) + adenosine (10^{-3}M)	7·9 ± 0·8
Theophylline + adenosine + cyclic AMP (4×10^{-5}M)	15 ± 0·4
Theophylline + adenosine + cyclic AMP (2×10^{-4}M)	21 ± 2
Theophylline + adenosine + cyclic AMP (1×10^{-3}M)	33 ± 0·3
Theophylline + adenosine + cyclic AMP (5×10^{-3}M)	34 ± 0·5

Fat cells were incubated for 1 hr. Each value is the mean of four experiments.

DISCUSSION

Acute alterations in physiological and biochemical activities of many tissues are controlled by neurohormones released from peripheral nerve endings. An understanding of the manner in which a neurophysiological message—the release of a neurohormone—is translated into a biochemical message is of outstanding importance. In studying such a problem, drugs that depress or stimulate the activity of its target organs are of unique value. A great many studies have been made of drugs that increase or reduce the amount of the neurohormone released at nerve endings, or prevent its access to receptor sites. The discovery that certain sympathetic functions may be controlled through cyclic 3′,5′-AMP makes it important to study the action of drugs on isolated cells. Since isolated fat cells are free of nerve endings and catecholamines, they are particularly useful in studies of drugs that affect the discrete biochemical events through which norepinephrine increases the mobilization of FFA.

In studying this mechanism, it is pertinent to establish whether the sympathetic

nervous system regulates FFA output through changes in cyclic 3′,5′-AMP. To support this view, several criteria must be met:

(1) Adipose tissue cells should contain an enzyme that catalyzes the synthesis of cyclic 3′, 5′-AMP. It is now firmly established that rat adipose tissue contains adenyl cyclase.[8, 17]

(2) Adipose tissue should contain an enzyme that readily inactivates the nucleotide. Our results indicate the presence of considerable phosphodiesterase activity.

(3) The degree of lipolytic activity in adipose tissue should be related to the concentration of cyclic 3′,5′-AMP. It is now established that cyclic 3′,5′-AMP is present in adipose tissue and that an increased level of the nucleotide is associated with an increase in lipolytic activity.[5, 8, 9]

(4) The sympathetic transmitter should be capable of stimulating the formation of cyclic 3′,5′-AMP and of evoking a lipolytic response. Catecholamines increase the formation of cyclic 3′,5′-AMP and elicit a marked lipolytic response in adipose tissue.[5, 17]

(5) Substances that inhibit the inactivation of cyclic 3′,5′-AMP should evoke a lipolytic response. Theophylline in concentrations that block phosphodiesterase cause the accumulation of cyclic 3′,5′-AMP and the stimulation of lipolysis.[8, 9]

(6) Cyclic 3′,5′-AMP should exert a lipolytic response. This important criterion has been difficult to satisfy, presumably because of the slow influx of the nucleotide into fat cells together with its rapid rate of inactivation. These obstacles have been overcome by the use of theophylline. This substance, in a concentration producing only a slight lipolytic response by itself, protects enough of the cyclic 3′,5′-AMP diffusing into fat cells to activate lipolysis to a maximal extent.

Since theophylline evokes considerable lipolysis, it may be inferred that cyclic 3′,5′-AMP is formed continuously in fat cells and that sympathetic stimulation merely increases the rate of synthesis by activating adenyl cyclase.[8, 9] According to this view, lipolytic activity is governed by the concentration of cyclic 3′,5′-AMP. Since this level depends on the relative rates of synthesis and inactivation, lipolysis may be increased by stimulating the activity of adenyl cyclase or depressing that of phosphodiesterase. The response to norepinephrine is limited, not by the capacity of the lipase system but by that of adenyl cyclase, and maximal effects are attained when adenyl cyclase is fully activated. On the other hand, when phosphodiesterase is blocked by theophylline, the maximal lipolysis is considerably greater than that attained by norepinephrine. In this case the degree of lipolysis is not limited by the rate of cyclic 3′,5′-AMP synthesis but by the capacity of the lipase system. Thus, the maximal effect attained by the combined actions of norepinephrine and theophylline is no greater than that attained by theophylline alone.

Of particular importance in this regard are results showing that cyclic 3′,5′-AMP elicits a lipolytic response in the presence of a small concentration of theophylline, The maximal response produced by cyclic 3′,5′-AMP is equal to that attained by a high concentration of theophylline; furthermore, the response of theophylline is not enhanced by high concentrations of nucleotide. It may be inferred, therefore, that the actions of both substances are limited by the same step—the complete activation of the lipase system.

The question arises why the release of FFA or glycerol from isolated fat cells is much more rapid than that from minces or whole fat pads. For example, the optimum

output of FFA is more than four times higher from isolated cells than from adipose tissue. A possible explanation for the more rapid efflux of FFA is the greater surface area of isolated fat cells in contact with the medium. In agreement with this view are results by Ho and Meng[18] showing that the FFA production is 2·5 times greater from perfused epididymal fat pads than from incubated tissue. These results suggest that high cellular concentrations of FFA inhibit the hydrolysis of triglycerides.[19]

Since ACTH and norepinephrine produce almost the same maximal lipolytic responses and are potentiated by theophylline to the same degree, the action of both substances is presumably mediated through cyclic 3′,5′-AMP. Calculation of the molar concentrations at which these hormones produce one half their maximal activity show that, mole for mole, ACTH is 100 times more active than norepinephrine.

In conclusion, the increased mobilization of FFA after sympathetic stimulation is caused by the activation of adenyl cyclase. But it should be emphasized that a number of steps may lie between stimulation of adenyl cyclase and activation of adipose tissue lipase. By analogy with the phosphorylase system, cyclic 3′,5′-AMP may activate a kinase which in turn converts inactive to active lipase.

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